Armadillo homologues in *Dictyostelium discoideum*

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Abstract

Armadillo family proteins are present throughout the animal kingdom. In fruit flies, frogs, fish and mammals these proteins are essential for correct embryonic patterning via the Wnt/Wingless signal transduction pathway, acting downstream of glycogen synthase kinase-3 (gsk3). In addition, the same proteins are essential for regulated cell to cell adhesion. These proteins must be tightly controlled to prevent the growth and metastasis of tumours in the adult organism. However, the relationship between their developmental and adhesive role is not well understood at present.

The cellular slime mould *Dictyostelium discoideum* is a model system in which to study multicellular development and cell adhesion. The *Dictyostelium* homologue of glycogen synthase kinase-3, gskA, is essential for the correct proportioning of cell types during development. The downstream targets of gskA remained to be discovered at the start of this work. I have isolated an Armadillo family gene, *aardvark* (*aar*), in *Dictyostelium* and created a knock-out mutant. The mutant phenotype demonstrates that *aar* has an important role in the later stages of development. Aardvark is crucial for defining the final structure of the fruiting body, particularly the assembly of the stalk tube and its associated cell-cell adhesion junctions. Perturbation of this structure in the *aar*$^-$ mutant leads to a duplication of the developmental axis. Aar also has a cell-autonomous role, promoting the terminal differentiation of stalk and spore cells.

Furthermore, I present evidence that two other Armadillo homologues are also present in *Dictyostelium*. These proteins are developmentally regulated and their levels and distribution are altered in a *gskA*$^-$ mutant.

This thesis suggests that the molecular control of pattern formation and cell to cell adhesion may be very highly conserved throughout evolution and that in *Dictyostelium*, as in animals, Armadillo homologues act to coordinate morphogenesis and cell identity.

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Chapter 1

Introduction

1.1 The problem: multicellular development

The problem of pattern formation in multicellular organisms has fascinated biologists for two thousand years. Aristotle's observations of the developing chick embryo were perhaps the first recorded instance of the study of development, and many major advances were made by embryologists in the late 19th century. Multicellular development involves the generation of cellular diversity. The fundamental question is how one cell, or a group of initially identical cells, forms a patterned adult organism, with many different cell types all in the correct place. Many species have been used as experimental models to address this question and many of the principles and molecules involved in development are highly conserved across species.

Cells acquire a specific fate within an organism, such as muscle or nerve, by a process termed differentiation. However, differentiation must be coupled to cell sorting and cell movement for an organism to acquire its final shape and pattern. This process is referred to as morphogenesis: cells must move in a co-ordinated manner to form tissues and organs in the correct place. Morphogenesis necessarily involves communication between cells and also regulated cell-cell adhesion; cells possess surface molecules allowing them to adhere together in an organised fashion to generate a variety of distinct patterns. My interest is in understanding how differentiation and regulated cell adhesion work together within a developing organism: if and how the processes are coupled is not well understood. The intensively studied Wnt/Wingless signalling pathway impinges on this problem as one of its components, ß-catenin/Armadillo, regulates gene expression whilst also being an essential component of intracellular adhesion junctions.

A combination of genetics in *Drosophila*, *C. elegans*, mammals and, more recently, zebrafish, and of embryology and cell biology in amphibia and echinoderms, has established that the Wnt/Wingless pathway has a conserved developmental function in metazoa. However many of the species studied are extremely complex with a huge number of cell types, and hence interactions, to dissect. Many knockouts of genes that may be involved in early patterning events are lethal, because these genes are also required for cell growth, and are hence developmentally uninformative. Organisms with well established genetics, such as *Drosophila*, are difficult to manipulate biochemically, thus complicating the study of protein interactions. Conversely, *Xenopus* can be grafted, transplanted, injected and ground up for large-scale

biochemistry with ease, but, with a tetraploid genome, it has only recently become possible to carry out genetic ablations and loss-of function studies.

Dictyostelium discoideum is a multicellular organism, in which the study of pattern formation is relatively simple. Upon starvation, free living, unicellular amoebae coalesce to form a multicellular structure, in a process that involves regulated cell adhesion, cell movements and differentiation of a number of cell types. *Dictyostelium*, being haploid, is a genetically tractable organism that can also be grown in large quantities for biochemical study. The ability to perform targeted gene ablations in *Dictyostelium* is used in conjunction with a random insertion mutagenesis approach (Kuspa and Loomis, 1992). As growth does not occur during development mutants can be screened for specific developmental phenotypes, from which the defective gene sequence can be recovered relatively simply.

Dictyostelium undergoes differentiation in conjunction with regulated cell adhesion and possesses a homologue of one Wnt signalling component, gskA, which regulates cell fate. My hypothesis is that a Wnt-like signalling system could operate in a non-metazoan eukaryote and I hope to use this simple system to investigate the relationship between a developmental signalling pathway and regulated cell-cell adhesion.

1.2 Conserved developmental signalling molecules in metazoa

1.2.1 Overview of the Wnt/Wingless signalling pathway

Wnts form a large multigene family, encoding secreted glycoprotein signalling molecules. The founding member, murine *int-1*, was discovered as a proto-oncogene containing a retroviral insertion in mammary tumours (Nusse and Varmus, 1982). Around twenty vertebrate *wnt* genes now exist, some of which have counterparts in *Drosophila* and *C. elegans* (Cadigan and Nusse, 1997). Many Wnts have functions at different times in development and in various tissues, as shown by genetic loss-of-function studies in mouse, *Drosophila* and *C. elegans* (Cadigan and Nusse, 1997; McMahon and Bradley, 1990). This thesis focuses on components downstream from the Wnt-1 class of signals. Other Wnts operate by distinct mechanisms, such as the Wnt-5A class affecting cell movement in *Xenopus* and mouse (Torres *et al.*, 1996; Yamaguchi *et al.*, 1999), possibly via regulation of heterotrimeric G-protein/protein kinase C/inositol signalling (Slusarski *et al.*, 1997).

The proteins of the Wnt-1 class signal transduction pathway are known to be fundamental for the early patterning events in metazoa. One of the hallmarks of

vertebrate Wnts of the Wnt-1 class is their ability to induce an ectopic axis when injected into *Xenopus* embryos, or to rescue the primary axis of embryos ventralised by UV (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol *et al.*, 1991). How do these Wnts exert their effect? Many elegant genetic loss-of-function studies have defined the molecular pathway transducing Wnt-1 signals. Mutants of Wingless (Wg), the *Drosophila* Wnt-1 homologue, display alterations in embryonic segmental pattern (Nusslein-Volhard and Wieschaus, 1980): each embryonic segment has its posterior denticle-free cuticle replaced by a mirror image duplication of the anterior denticle pattern. Mutations in other loci, namely *porcupine* (*porc*), *dishevelled* (*dsh*) *armadillo* (*arm*) and *pangolin* (*pan*), produce the same segment polarity phenotype (Cadigan and Nusse, 1997). Maternal *zeste-white-3/shaggy* (*zw3/sgg*) mutants have the opposite segment polarity phenotype, i.e. no denticles, with the anterior of each segment consisting of naked cuticle (Siegfried *et al.*, 1990). *porc* is required for the secretion of the Wg signal, while *dsh*, *zw3/sgg*, *arm* and *pan* are required, in that order, in the cell receiving the Wg signal (Brunner *et al.*, 1997; Klingensmith and Nusse, 1994; Noordermeer *et al.*, 1994b; Peifer *et al.*, 1994c; Siegfried *et al.*, 1994; Wieschaus and Riggleman, 1987). A variety of mutant alleles have demonstrated that components of *Drosophila* Wg signalling are used to define cell fates at many points in development, and in many tissues (Neumann and Cohen, 1996; Peifer *et al.*, 1991; Struhl and Basler, 1993). Although not shown to function in segment polarity, *Drosophila frizzled* (*fz*) is required to establish cellular polarity in tissues and interacts genetically with *dsh* (Orsulic and Peifer, 1996a). Genetic interactions between Wnts and Fzs also exist in *C. elegans* (Han, 1997; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). Another Wnt pathway member, first uncovered by mouse genetics, is Axin, the product of the *fused* locus (Zeng *et al.*, 1997). *fused* mutants have a duplicated embryonic axis, similar to the effect of injected *wnt-1* mRNA in *Xenopus*. Subsequently, *Drosophila* Axin has been demonstrated to inhibit Wg signalling and maternal *D-Axin* mutants show an embryonic naked cuticle phenotype (Hamada *et al.*, 1999; Willert *et al.*, 1999a).

Vertebrate homologues of all the originally identified *Drosophila* Wg signalling components exist: frizzleds, dvl and Xdsh (mouse and Xenopus *dsh* respectively), gsk3ß (*zw3*), ß-catenin (*arm*), and LEF-1/TCF (*pan*) (Brunner *et al.*, 1997; McCrea *et al.*, 1991; Peifer *et al.*, 1992; Peifer and Wieschaus, 1990; Siegfried *et al.*, 1990; Sokol *et al.*, 1995; Sussman *et al.*, 1994; Wang *et al.*, 1996). Like Wnt-1, dsh, ß-catenin, LEF-1/TCF and a dominant negative form of gsk3ß from a variety of species can cause axis duplication (duplication of dorsal structures) in *Xenopus* or zebrafish, or rescue

UV-ventralised *Xenopus* embryos when expressed ectopically (Dominguez *et al.*, 1995; Funayama *et al.*, 1995; He *et al.*, 1995; Kelly *et al.*, 1995; McCrea *et al.*, 1993; Pierce and Kimelman, 1995; Sokol *et al.*, 1995). Depletion of maternal *ß-catenin* mRNA from *Xenopus* embryos caused a failure to form dorsal structures (Heasman *et al.*, 1994). Injection of mouse Axin or a dominant negative form of Xdsh suppresses Wnt-induced axis formation in *Xenopus* (Sokol *et al.*, 1995; Zeng *et al.*, 1997). In *C. elegans*, Wnt, Porc, Fz, zw3, Arm and Pan homologues act to promote endoderm formation (Rocheleau *et al.*, 1997; Schlesinger *et al.*, 1999; Thorpe *et al.*, 1997) and Wnt, Fz and Arm homologues function during later developmental stages (Eisenmann *et al.*, 1998; Han, 1997; Maloof *et al.*, 1999). Thus the developmental function of these molecules is conserved between vertebrates and invertebrates. The proteins and their relationships with one another will be discussed in detail in the following sections.

1.2.2 Wnt receptors

For many years a cell surface receptor for Wnt signals remained elusive. A large family of related but divergent 7-transmembrane proteins homologous to Fz (Vinson *et al.*, 1989) was cloned by PCR from a variety of metazoa (Wang *et al.*, 1996). The genetic interactions previously established between *fz*, *wg* and *dsh*, plus the fact that the Fz family seemed large enough to accommodate the equally large number of existing Wnt proteins, led the authors, like others before them, to propose that Frizzleds could act as Wg receptors (Krasnow *et al.*, 1995; Wang *et al.*, 1996).

Functional interactions between Wnts and Fzs are now well established. *Drosophila* S2 tissue culture cells are unresponsive to Wg signal unless transfected with *Drosophila Fz2* (*Dfz2*) mRNA (Bhanot *et al.*, 1996). These Dfz2-expressing cells bind Wg on their surface, as can human cells transfected with *Dfz2*. Wg can also bind Fz and a subset of mouse Frizzleds expressed in S2 cells. The extracellular domain of Dfz2, containing ten invariant cysteines, is necessary and sufficient for Wg binding (Bhanot *et al.*, 1996). Overexpression of Dfz2 mimics the Wg signal (Ruel *et al.*, 1999) and a similar functional assay in *Xenopus* animal cap explants showed that a heterologously expressed rat Frizzled (rFz1) could recruit endogenous Xdsh and XWnt8 to the membrane and induce the expression of XWnt8 target genes (Yang-Snyder *et al.*, 1996). XWnt8 and Rfz1 acted synergistically in this process, but Rfz1 could not bind or be activated by XWnt5A, showing that Wnt and Fz family members have specificity in *vivo* (Yang-Snyder *et al.*, 1996).

Why do fly *fz* mutants not have a segment polarity phenotype like that of *wg* and *arm*, if Fz is a Wg receptor? The answer lies in functional redundancy between Fz and Dfz2 in the embryonic Wg signalling. The function of Fz in defining tissue polarity is unique: it requires *dsh* but not *wg* (Adler, 1992; Krasnow *et al.*, 1995) and the C-terminal region of *dsh* (not required for Wg function) activates a RhoGTPase/JNK pathway (Boutros *et al.*, 1998). However, zygotic *Dfz2* mutants show almost normal *wg* signalling in the embryo (Bhanot *et al.*, 1999; Muller *et al.*, 1999a), suggesting Fz can replace Dfz2 in this situation. Zygotic *fz/Dfz2* double mutants show reduced Wg signalling, and this can be eliminated completely by removing maternal *fz* as well: maternal *Dfz2* is not present in the embryo (Bhanot *et al.*, 1999; Muller *et al.*, 1999a). A similar situation exists later in development: *fz* and *Dfz2* both have similar neurogenic defects to *wg* but the phenotypes are not fully penetrant (Bhat, 1998). A double mutant has an increased, although not fully, penetrant phenotype, suggesting that other receptors may play a redundant role (Bhat, 1998). However, overexpression of either Fz or Dfz2, but not Wg, can rescue the embryonic segment polarity phenotype caused by eliminating both *Dfz2* and *fz* in the embryo, albeit with Dfz2 having better rescuing activity than Fz (Bhanot *et al.*, 1999). This suggests that Dfz2 and Fz are genuinely redundant in this process and that no other proteins are involved in the reception of Wg signal in the embryo (Bhanot *et al.*, 1999).

The situation has become more complex with the discovery of vertebrate extracellular antagonists that block Wnt/Fz signalling. Some of these have similarity to the extracellular, cysteine rich domain of Fz and are suggested to work by binding to Wnts and therefore preventing their interaction with the receptor (Bafico *et al.*, 1999; Leyns *et al.*, 1997; Lin *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997; Xu *et al.*, 1998). The inhibitors are spatially restricted in *Xenopus* embryos and thus their function may depend on the endogenous Wnt they encounter (Leyns *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997; Xu *et al.*, 1998). In addition, secreted Wnt-binding antagonists exist which are unrelated to the extracellular domain of Fz (Fedi *et al.*, 1999; Hseih *et al.*, 1999). Thus a whole level of Wnt regulation exists before Wnts even reach their receptors.

1.2.3 Cytoplasmic effectors of Wnt signalling

Dsh/Dvl is a cytoplasmic protein required in the receiving cell to transduce the Wnt/Wg signal (Klingensmith *et al.*, 1994; Theisen *et al.*, 1994). *Drosophila* Dsh is phosphorylated on serine or threonine by Wg signal (Yanagawa *et al.*, 1995). Receipt of

Wg signal leads to phosphorylation of a small pool of Dsh and its translocation to the membrane (Yanagawa *et al.*, 1995; Yang-Snyder *et al.*, 1996). Dvl-1 and Dvl-2 are part of a multiprotein complex in vertebrate cells. Axin and Dvl-1 bind to each other *in vitro* and *in vivo* (Kishida *et al.*, 1999) and Axin and Xdsh colocalise in *Xenopus* embryos (Fagotto *et al.*, 1999; Yamamoto *et al.*, 1999). Axin and Dvl-2 colocalise in the cytoplasm of mammalian cells and a membrane-tethered form of Axin can recruit Dvl-2 to the membrane (Smalley *et al.*, 1999). However, Dsh has functions independent of Wnt/Wg signalling. As well as its participation in the tissue polarity pathway with Fz and JNK (Boutros *et al.*, 1998; Krasnow *et al.*, 1995), Dsh also mediates inhibitory crosstalk between Wg and Notch signalling events during neurogenesis by binding directly to Notch (Axelrod *et al.*, 1996).

Another component of the Dsh/Axin complex is gsk3ß (Hart *et al.*, 1998; Ikeda *et al.*, 1998; Itoh *et al.*, 1998; Sakanaka *et al.*, 1998; Willert *et al.*, 1999a; Yamamoto *et al.*, 1998). Studies of mutant *Xenopus* Axins have shown that the axis-inhibitory ability of Axin is dependent on its binding to gsk3ß (Itoh *et al.*, 1998). gsk3ß is a serine/threonine protein kinase, homologues of which are found in a wide range of eukaryotic species, such as yeasts, *Dictyostelium*, *Drosophila*, echinoderms, *C. elegans*, vertebrates and plants (Bianchi *et al.*, 1994; Emily-Fenouil *et al.*, 1998; Harwood *et al.*, 1995; Pay *et al.*, 1993; Pierce and Kimelman, 1995; Schlesinger *et al.*, 1999; Siegfried *et al.*, 1990; Welsh *et al.*, 1996). gsk3ß has many cellular targets including transcription factors, signalling proteins and cytoskeletal proteins and is involved in metabolism, growth, cell morphology, meiosis and cell cycle arrest (Delcommenne *et al.*, 1998; Fisher *et al.*, 1999; Lucas *et al.*, 1998; Puziss *et al.*, 1994; Welsh *et al.*, 1996).

Animal homologues of gsk3ß act in the Wnt/wg signalling pathway. The *Drosophila* homologue of gsk3ß, *zw3/sgg* (Siegfried *et al.*, 1992; Siegfried *et al.*, 1990) is a segment polarity gene as discussed previously and also inhibits the adoption of dorsal fates in the developing leg (Diaz-Benjumea and Cohen, 1994). Wg signal antagonises zw3 activity (Diaz-Benjumea and Cohen, 1994; Siegfried *et al.*, 1990). A catalytically inactive (dominant negative) mutant form of human or *Xenopus* gsk3ß, when injected ventrally into *Xenopus* embryos, is a potent inducer of ectopic axes in early *Xenopus* embryos (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995). Conversely dorsal injection of wild type gsk3ß blocks formation of dorsoanterior structures (He *et al.*, 1995; Pierce and Kimelman, 1995). Similarly, a dominant negative sea urchin gsk3ß injected into the egg before fertilisation causes embryonic cells to adopt more vegetal fates, whereas overexpression of wild type gsk3ß

gives rise to an excess of animal structures (Emily-Fenouil *et al.*, 1998). As for *zw3* and *dsh* in *Drosophila*, Xgsk3 acts downstream of Xdsh (Dominguez *et al.*, 1995) and a dominant negative form of gsk3ß blocks the function of Dvl-2 in mammalian cells (Smalley *et al.*, 1999). A *C. elegans* gsk3ß homologue functions downstream of a Wnt signal during endoderm formation. However, unlike the *Drosophila* and vertebrate kinases, worm gsk3ß appears to be activated by Wnt, but the mechanism for this is unknown (Schlesinger *et al.*, 1999).

The inhibition of gsk3ß by Wg was demonstrated biochemically by measuring the activity of endogenous gsk3ß in a mouse cell line challenged with Wg-conditioned medium from *Drosophila* S2 cells (Cook *et al.*, 1996). The Wg-mediated inhibition of gsk3ß required serine/threonine phosphorylation of gsk3ß and PKC activation, being sensitive to protein kinase C inhibitors and activators (Cook *et al.*, 1996). Overexpression of dsh inhibits zw3 activity and causes its serine/threonine phosphorylation in *Drosophila* cell culture (Ruel *et al.*, 1999). Lithium ions have been shown to inhibit the activity of gsk3 (Klein and Melton, 1996; Stambolic *et al.*, 1996) and mimic the effects of Wnt/Wg signalling and/or gsk3 inhibition in *Xenopus*, *Drosophila*, sea urchins, ascidians mammals and *Dictyostelium* (Emily-Fenouil *et al.*, 1998; Hedgepeth *et al.*, 1997; Klein and Melton, 1996; Logan *et al.*, 1999; Lucas *et al.*, 1998; Maeda, 1970; Roeser *et al.*, 1999; Stambolic *et al.*, 1996; Wikramanayake *et al.*, 1998; Yoshida *et al.*, 1998). zw3 phosphorylates *Drosophila* Axin (D-Axin) *in vitro* (Ruel *et al.*, 1999). Moreover, a Wnt signal leads to dephosphorylation of Axin *in vivo* and Wg inhibits the interaction of zw3 with D-Axin (Ruel *et al.*, 1999; Willert *et al.*, 1999b). Furthermore, Dvl-1 inhibits the gsk3ß-dependent phosphorylation of Axin in a non-competitive manner *in vitro* (Yamamoto *et al.*, 1999); this is due to the recruitment of an inhibitor of gsk3ß, GBP/Frat-1, to the complex *in vivo*. GBP is required for formation of the endogenous dorsoventral axis in *Xenopus*; when GBP is antisense-depleted from the oocyte only ventral tissue forms, whilst injected GBP can induce an ectopic axis (Yost *et al.*, 1998). Mammalian Frat-1 and *Xenopus* GBP are present in a complex with Axin, dvl/Xdsh, and gsk3ß/Xgsk3; Wnt-1 causes dissociation of the complex (Li *et al.*, 1999). GBP/Frat-1 remains bound to gsk3ß and thus may drive the dissociation by competing with Axin (Li *et al.*, 1999).

1.2.4 A downstream target of gsk3ß/zw3 in Wnt/Wg signalling.

As well as phosphorylating Axin, gsk3ß/zw3 has another target in the Wnt/Wg pathway, ß-catenin/Armadillo. ß-catenin is present in a ternary complex with gsk3ß and

Axin (Behrens *et al.*, 1998; Hart *et al.*, 1998; Ikeda *et al.*, 1998; Sakanaka *et al.*, 1998; Willert *et al.*, 1999a; Yamamoto *et al.*, 1998). Direct *in vivo* phosphorylation of ß-catenin by gsk3ß has been difficult to demonstrate, although much indirect and *in vitro* evidence exists that Wnt/Wg signalling, via inhibition of gsk3ß/zw3, leads to a decrease in ß-catenin/Arm phosphorylation.

arm/zw3 double mutant embryos have an *arm* phenotype placing *arm* downstream of *zw3* (Klingensmith and Nusse, 1994; Noordermeer *et al.*, 1994b; Siegfried *et al.*, 1994). Extending this finding, gene dosage experiments demonstrated that zygotic *arm/zw3* heterozygotes develop normally (Peifer *et al.*, 1994c). Levels of Arm protein are increased in the areas of the fly embryo (the anterior cells in each segment) that express *wg*, and alteration of *wg* mRNA distribution redistributes Arm accordingly (Noordermeer *et al.*, 1994a; Riggleman *et al.*, 1990). Wg modulates Arm distribution post-transcriptionally since zygotic *arm* mRNA is evenly distributed throughout the embryo (Riggleman *et al.*, 1990). *In vitro* translated Arm is 7-10kDa smaller than Arm from cell lysates *in vivo*, suggesting modification of the protein occurs (Riggleman *et al.*, 1990). Arm is phosphorylated on serine, threonine and tyrosine residues, as shown by ^{32}P labelling in conjunction with serine/threonine phosphatase inhibitors and immumoprecipitation of Arm with anti-phosphotyrosine antibodies (Peifer *et al.*, 1994b). The major cause of the band shift of Arm seen on SDS-PAGE is due to serine and threonine phosphorylation. In *wg* embryos there is a specific decrease in unphosphorylated Arm (Peifer *et al.*, 1994b). Unphosphorylated Arm is soluble in non-ionic detergents suggesting a cytoplasmic location. Conversely, maternal *zw3* mutant embryos have a greatly reduced level of phosphorylated Arm early in embryogenesis (Peifer *et al.*, 1994b). Confocal microscopic analysis of Arm distribution in embryos showed that in *wg* embryos all Arm was membrane-associated and phosphorylated, whereas in *zw3* embryos Arm was largely cytoplasmic and unphosphorylated (Peifer *et al.*, 1994c). A model was proposed whereby Wg signal acts to repress Arm phosphorylation by zw3 on serine/threonine, leading to an increased level of unphosphorylated, cytoplasmic Arm. It is this pool of stable, cytoplasmic Arm which acts to transduce the Wg signal, independently from the membrane-associated, phosphorylated Arm (Peifer *et al.*, 1994c).

Does zw3/gsk3ß phosphorylate Arm/ß-catenin directly? *Xenopus* gsk3ß can phosphorylate ß-catenin *in vitro* (Yost *et al.*, 1996). There are four conserved N-terminal serines in ß-catenin and Armadillo which are putative gsk3ß phosphorylation sites (Peifer *et al.*, 1994b). If these are deleted or mutated in ß-catenin its gsk3ß-

mediated phosphorylation is reduced, but not abolished (Yost *et al.*, 1996), indicating the presence of other gsk3ß sites in the N-terminus of ß-catenin (Yost *et al.*, 1996) and the C-terminus of Arm (Peifer *et al.*, 1994b). In *Xenopus* embryos, injected wild type ß-catenin is phosphorylated by co-injected Xgsk3 to a much greater extent that ß-catenin with mutated N-terminal serines; injection of dominant negative Xgsk3 reduces the level of wild type ß-catenin phosphorylation down to that of the mutant ß-catenin. Interestingly, N-terminally deleted ß-catenin has more potent axis inducing ability than wild type in Zebrafish and *Xenopus* (Kelly *et al.*, 1995; Yost *et al.*, 1996) and axis induction by the deletion mutant is not suppressed by co-injection of wild type Xgsk3 (Yost *et al.*, 1996). Similarly, constitutive expression of an N-terminal truncation of Arm in the *Drosophila* embryo or wing imaginal disc leads to a *zw3*-like phenotype (Zecca *et al.*, 1996), and overexpression of N-terminally mutated ß-catenin in the sea urchin mimicked the effect of Li^+ treatment (Wikramanayake *et al.*, 1998). Furthermore, Axin/D-Axin potentiates the phosphorylation of ß-catenin/Arm by gsk3ß/zw3 *in vitro* (Ikeda *et al.*, 1998; Ruel *et al.*, 1999). These results suggest that the N-terminal serines are important for gsk3ß-mediated phosphorylation of ß-catenin/Arm *in vivo*.

What is the biochemical effect of ß-catenin phosphorylation? Pulse-chase analysis showed that N-terminal mutants of ß-catenin are more stable than wild type proteins in *Xenopus* embryos (Yost *et al.*, 1996) and mammalian cell lines (Munemitsu *et al.*, 1996). Furthermore, injection of dominant negative Xgsk3 led to an increase in the stability of wild type, but not mutant ß-catenin (Yost *et al.*, 1996), thus confirming the previously suggested model for Arm regulation (Peifer *et al.*, 1994c). Axin also decreases the half-life of full length but not N-terminally deleted ß-catenin *in vivo* (Hart *et al.*, 1998; Kishida *et al.*, 1998). N-terminally deleted ß-catenin has been shown to have increased stability in every animal system tested: in epithelial cells (Barth *et al.*, 1997), intestine (Wong *et al.*, 1998), skin (Gat *et al.*, 1998; Noramly *et al.*, 1999; Zhu and Watt, 1999), melanoma cells (Rubinfeld *et al.*, 1997) and apoptotic cells, where it is naturally generated by caspases (Brancolini *et al.*, 1997). Thus the N-terminal phosphorylation and destabilisation of ß-catenin may be a general *in vivo* mechanism for its regulation. ß-catenin levels must be kept low *in vivo* in the absence of signals as misexpression of stable ß-catenin can lead to deregulated proliferation, apoptosis and changes in adhesion and cell fate (Gat *et al.*, 1998; Noramly *et al.*, 1999; Wong *et al.*, 1998; Zhu and Watt, 1999) which can contribute to oncogenesis (Korinek *et al.*, 1997; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997). Wnt signalling also upregulates

phosphorylated Axin, which binds ß-catenin, while unphosphorylated Axin does not (Willert *et al.*, 1999b) although this was not observed *in vitro* (Ikeda *et al.*, 1998). This suggests that Axin is a major target of gsk3ß and destabilisation of ß-catenin is caused by its dissociation from the Axin complex (Willert *et al.*, 1999b).

Additional data suggests the regulation of Arm/ß-catenin is complex. *zw3*⁻ embryos have reduced tyrosine-phosphorylated Arm (Peifer *et al.*, 1994b). In addition, okadaic acid treatment of *zw3* embryo extracts restores Arm phosphorylation on serine/threonine and the authors propose that zw3 could be acting indirectly on Arm by regulating the activity of a phosphatase. Similarly, overexpression of a regulatory subunit of protein phosphatase 2A (PP2A) decreases the levels and signalling ability of ß-catenin (Seeling *et al.*, 1999). Indeed, PP2A binds to and dephosphorylates Axin (Hsu *et al.*, 1999; Willert *et al.*, 1999b). A mutant Axin without the PP2A binding region ventralises *Xenopus* embryos, and this form of Axin is particularly potent at ventralising (Fagotto *et al.*, 1999), due to ß-catenin being constantly in a phosphorylated (unstable) state. However, Li^+ inhibition of gsk3ß leads to dephosphorylation of Axin suggesting the phosphatase activity is constitutive rather than being regulated by Wnt (Willert *et al.*, 1999b).

1.2.5 Regulating the stability of Arm/ß-catenin

How does the proposed phosphorylation of ß-catenin/Arm by gsk3ß/zw3 lead to an increase in its stability? The Adenomatous Polyposis Coli (APC) tumour suppressor gene product (Groden *et al.*, 1991; Joslyn *et al.*, 1991) was detected in a ternary complex with ß-catenin, gsk3ß and Axin or a related protein, Conductin, in mammalian cells (Behrens *et al.*, 1998; Hart *et al.*, 1998). This extended earlier observations that APC, ß-catenin and gsk3ß formed a complex in human colon carcinoma cells (Munemitsu *et al.*, 1996; Rubinfeld *et al.*, 1996; Rubinfeld *et al.*, 1993; Rubinfeld *et al.*, 1995; Shibata *et al.*, 1994; Su *et al.*, 1993).

APC is a large (~300kDa) protein with multiple partners (Behrens *et al.*, 1998; Berrueta *et al.*, 1998; Hart *et al.*, 1998; Matsumine *et al.*, 1996; Rubinfeld *et al.*, 1993; Su *et al.*, 1993) the function of which is largely unknown. Expression of wild type APC in colon carcinoma cells that contain only truncated mutant APC reduces the level of cytoplasmic ß-catenin (Easawaran *et al.*, 1999; Munemitsu *et al.*, 1995). Wild type APC increases the rate of degradation of ß-catenin rather than inhibiting its synthesis: cytoplasmic ß-catenin normally has a half-life of ~0.5 hours, (Munemitsu *et al.*, 1995). Truncated APC in colon carcinoma cells can bind ß-catenin but cannot promote its

degradation, suggesting that high levels of cytosolic ß-catenin contribute to the transformed phenotype of these cells (Rubinfeld *et al.*, 1996). Moreover, expression of Wnt-1 in two mammalian cell lines increases the half life of both APC/ß-catenin complexes and a pool of "monomeric" ß-catenin (Papkoff *et al.*, 1996). Exogenously expressed ß-catenin is only stable in cells also expressing Wnt-1 and overexpression of the central region of APC opposes this stabilisation of ß-catenin by Wnt-1 (Papkoff *et al.*, 1996). Thus wild type APC was proposed to function in keeping the turnover rate of cytosolic ß-catenin high and its level low. Wnt signalling somehow inhibits the turnover of ß-catenin allowing a cytosolic "signalling" pool to accumulate. The central domain of *Drosophila* APC (dAPC) binds Arm and downregulates ß-catenin in a mammalian cell line, albeit inefficiently (Hayashi *et al.*, 1997). An increase in Arm could mimic a *dAPC* defect in the developing Drosophila retina and the *dAPC* defect could be rescued by lowering Arm levels in gene dosage experiments (Ahmed *et al.*, 1998). Thus dAPC in some situations functions to antagonise Arm, supporting the data from colon carcinoma cells.

However, the exact function of APC in Wnt signalling and the regulation of ß-catenin stability is not clear. *Drosophila* APC (dAPC) has no effect on embryonic segment polarity or Arm distribution (Hayashi *et al.*, 1997). *Xenopus* APC (XAPC), which binds ß-catenin, induced an ectopic axis when expressed ventrally in four-cell embryos without changing the protein levels of ß-catenin (Vleminckx *et al.*, 1997). The ß-catenin binding domain alone could also induce an ectopic axis, although an N-terminal XAPC fragment that could also bind ß-catenin could not cause axis duplication Thus sequestration of ß-catenin was not the cause of axis induction (Vleminckx *et al.*, 1997). If APC was acting simply to bind and degrade ß-catenin then suppression of dorsal axis formation would have been expected when APC was overexpressed; the authors suggested a positive signalling role for APC during early embryogenesis. Likewise in *C. elegans*, RNA-mediated inhibition of *apr-1*, an APC homologue, produced the same, fully penetrant phenotype of mesoderm differentiation in the place of gut endoderm as did inhibition of *wrm-1*, a ß-catenin homologue (Rocheleau *et al.*, 1997). The authors placed Apr-1 in a pathway parallel to a Wnt signal, positively acting on Wrm-1 activity. If and how Wrm-1 is degraded in *C. elegans* is unknown. Axin potentiates the phosphorylation and degradation of ß-catenin by gsk3ß *in vitro* (Hart *et al.*, 1998; Ikeda *et al.*, 1998) and phosphorylation of APC *in vivo* (Fagotto *et al.*, 1999). In addition, excess Axin can degrade ß-catenin without the help of APC (Itoh *et al.*, 1998; Li *et al.*, 1999). The vast overexpression of *Xenopus* APC caused by

mRNA injection could be sequestering endogenous Axin, preventing gsk3ß-mediated phosphorylation of ß-catenin and allowing deregulated dorsal signalling. Perhaps APC is acting to regulate assembly of the ternary complex *in vivo*: The importance of APC homologues in regulating Axin-mediated ß-catenin degradation may depend on cellular context. Dissecting the situation may be further complicated by the discovery of a second APC homologue in both flies and mammals (van Es *et al.*, 1999).

1.2.6 Degradation of ß-catenin

If a multiprotein complex in the cytoplasm functions to regulate the stability of Arm/ß-catenin, then how are unstable forms of ß-catenin actually degraded? A combination of biochemical and genetic studies has again provided the answer. The stability of ß-catenin, but not the more distantly related p120 (see section 1.6), is increased by inhibitors of proteasome-mediated proteolysis in a variety of mammalian cell lines (Aberle *et al.*, 1997; Orford *et al.*, 1997; Salomon *et al.*, 1997; Simcha *et al.*, 1998). Proteins are targeted for degradation by the proteasome by addition of ubiquitin moieties (Ciechanover, 1998). The inhibition of proteolysis allowed the detection of multi-ubiquitinated forms of ß-catenin by SDS-PAGE; indeed ubiquitin can be coimmunoprecipitated with ß-catenin (Aberle *et al.*, 1997). Ubiquitination of ß-catenin was inhibited by mutation of its N-terminal gsk3ß sites, by addition of Li^+ or by addition of Wnt-1 signal, demonstrating that the likely cause of ß-catenin stabilisation was an inhibition of ubiquitination by Wnt signalling *in vivo*. (Aberle *et al.*, 1997; Orford *et al.*, 1997). One of the N-terminal gsk3 sites of ß-catenin coincides with a conserved ubiquitin-conjugation motif which is also found in other proteins known to be degraded by the proteasome (Aberle *et al.*, 1997; Orford *et al.*, 1997). Mutation of just one serine residue within this site inhibits ubiquitination of ß-catenin completely (Orford *et al.*, 1997). Furthermore, certain PKC inhibitors block the ubiquitination of ß-catenin (Easawaran *et al.*, 1999; Orford *et al.*, 1997). The PKC isoform involved is distinct from that regulating Wnt-inhibition of gsk3ß (Cook *et al.*, 1996), as gsk3ß activity remains unaffected by specific PKC inhibitors (Orford *et al.*, 1997) and Li^+ cannot inhibit ubiquitination of ß-catenin directly (Easawaran *et al.*, 1999).

A mutation in a *Drosophila slimb* gene allows the cell-autonomous accumulation of Arm protein and ectopic activation of Wg-responsive genes (Jiang and Struhl, 1998). The *Xenopus* homologue of Slimb, ßTrcp, suppresses *Xwnt-8*-induced axis duplication (Marikawa and Elinson, 1998). This suggests Slimb/ßTrcp is a negative regulator of Wnt signalling. A mutant ßTrcp without the conserved F-box

region induced an ectopic axis (Liu *et al.*, 1999; Marikawa and Elinson, 1998), which was suppressed by co-injection of Xgsk3 or Axin, but not Xdsh, showing that mutant ßTrcp probably induces an axis via accumulation of stable ß-catenin (Marikawa and Elinson, 1998). Slimb/ßTrcp homologues in yeast are known to target many proteins for ubiquitination and degradation. A mechanism may exist for allowing Slimb to specifically target Arm for ubiquitination (Jiang and Struhl, 1998). This is perhaps provided by regulating the subcellular location of Slimb/ßTrcp; since both ßTrcp and its mouse homologue, FWD1, are found in a complex with Axin, ß-catenin, APC and gsk3ß (Kitagawa *et al.*, 1999; Liu *et al.*, 1999). In addition, phosphorylation of ß-catenin on N-terminal serines is necessary and sufficient for recognition of ß-catenin by ßTrcp and its subsequent degradation (Kitagawa *et al.*, 1999; Liu *et al.*, 1999).

1.2.7 Transducing the Wg/Wnt signal: the role of ß-catenin in gene transcription

What are the cellular functions of stabilised Arm/ß-catenin during development? It is well established that the endpoint of Wg/Wnt signalling is a change in gene expression that brings about cell fate specification. The presence of developmentally regulated nuclear ß-catenin in vertebrate embryos provided the first clue as to how this was achieved: ß-catenin is present in the nuclei of prospective dorsal cells of *Xenopus* and mouse embryos (Larabell *et al.*, 1997; Roeser *et al.*, 1999; Schneider *et al.*, 1996). This domain of nuclear localisation is abolished in UV-ventralised embryos and expanded by Li^+ or by ectopic expression of dominant negative gsk3ß or Wnts (Larabell *et al.*, 1997; Roeser *et al.*, 1999; Schneider *et al.*, 1996; Yost *et al.*, 1996). The importance of ß-catenin signalling in early development has also been demonstrated in ascidians (Yoshida *et al.*, 1998) and in the sea urchin, where strong nuclear staining of ß-catenin was observed in vegetal cells (Logan *et al.*, 1999). This nuclear localisation was expanded by Li^+ treatment, with a concomitant increase in the number of endoderm and mesoderm precursor cells in the embryo (Logan *et al.*, 1999). Expression of ß-catenin was sufficient to re-specify ectodermal precursor cells as endoderm (Wikramanayake *et al.*, 1998). In *Drosophila*, nuclear localisation of Arm is essential for its signalling function, as membrane localised Arm transgenes cannot rescue the *arm* segment polarity defect (Cox *et al.*, 1999).

A breakthrough in the understanding of the functions of nuclear ß-catenin/Arm came with the discovery of its binding to the N-terminus of HMG-domain containing transcription factors of the LEF-1/TCF family (Behrens *et al.*, 1996; Brunner *et al.*, 1997; Huber *et al.*, 1996; Molenaar *et al.*, 1996; van de Wetering *et al.*, 1997). LEF-1,

XTCF3 and *Drosophila* TCF (dTCF/Pan) form a ternary complex containing ß-catenin/Arm and DNA containing LEF-1/TCF binding sites (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996; Riese *et al.*, 1997; van de Wetering *et al.*, 1997). This induces bending of the DNA in band shift assays (Behrens *et al.*, 1996). Ventral injection of LEF-1 can induce an ectopic axis in *Xenopus* and this effect is augmented by co-injection of ß-catenin (Behrens *et al.*, 1996; Huber *et al.*, 1996). However, despite being present endogenously in *Xenopus* embryos, injection of XTCF3 is unable to induce an axis, or augment the axis inducing ability of ß-catenin (Molenaar *et al.*, 1996). Injection of XTCF3 can cause the nuclear translocation of endogenous ß-catenin, and the ß-catenin/XTCF3 complex activates a TCF reporter gene (Molenaar *et al.*, 1996). LEF/TCFs lacking the ß-catenin binding domain or the HMG domain required for nuclear localisation act in a dominant negative fashion in *Xenopus*. These mutant LEF/TCFs ventralise embryos when injected dorsally, and suppress ß-catenin-mediated axis duplication and reporter gene activation (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996).

Epistasis experiments in *Drosophila* and *C. elegans* place LEF/TCFs downstream of Wg/Wnt and Arm/ß-catenin. Loss-of-function or dominant negative zygotic *pan* alleles have embryonic segment polarity phenotypes similar to those of *wg* and *arm* (Brunner *et al.*, 1997; van de Wetering *et al.*, 1997), and other *pan* alleles with *wg*-like phenotypes in imaginal discs have mutations which reduce binding of Pan to Arm (Brunner *et al.*, 1997). Arm repeats 3-8 mediate Arm binding to dTCF (van de Wetering *et al.*, 1997), in agreement with this region being essential for the Wg signalling function of Arm (Orsulic and Peifer, 1996b). The C-terminal region of Arm acts as a transactivation domain when fused to a heterologous protein (van de Wetering *et al.*, 1997) and the authors define Arm as a coactivator of TCF-mediated transcription. Conversely, *C. elegans* POP-1, a TCF homologue, may repress transcription during induction of gut endoderm (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). POP-1 is normally required to suppress endoderm formation (Lin *et al.*, 1995), and *pop-1* and *wrm-1* mutants have opposite phenotypes suggesting that a Wnt/ß-catenin signal negatively regulates POP-1. Indeed, nuclear localisation of POP-1 is reduced in the cell receiving the Wnt signal, suggesting that removal of nuclear POP-1 by Wrm-1 derepresses endoderm-specific gene activity (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). A second *C. elegans* transcription factor, SON1, may be acting as a transcriptional activator. *son1* mutants have an identical phenotype to *lin17* (Wnt) mutants, with certain cells adopting a glial, instead of a neuronal fate (Jiang and

Sternberg, 1999). Overexpression of POP-1 results in the same phenotype as *son1* and *lin17*, suggesting the two HMG proteins have opposite effects on transcription. The lin17/Wnt signal could be lifting transcriptional repression by POP-1, allowing SON1 to activate transcription of target genes (Jiang and Sternberg, 1999). An activatory role for ß-catenin/human TCF4 (hTCF4) in colon carcinoma and melanoma cell lines also exists: stable ß-catenin/hTCF4 complexes (due to the presence of mutant APC or ß-catenin) constitutively activated a reporter gene (Korinek *et al.*, 1997; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997). Furthermore, it appears that the ß-catenin/TCF complex could affect target gene transcription by interacting with the basal transcription machinery. GST-ß-catenin can pull down Pontin52, a putative DNA helicase from mammalian cells, in a complex also containing LEF-1; Pontin52 in turn is known to bind the TATA box binding protein (Bauer *et al.*, 1998).

How does ß-catenin localise to the nucleus? ß-catenin can dock on the nuclear membrane and be imported without LEF-1 (Fagotto *et al.*, 1998; Prieve and Waterman, 1999). This import is not the classical nuclear localisation signal-mediated import (Fagotto *et al.*, 1998; Prieve and Waterman, 1999; Sweet and Gerace, 1995). ß-catenin interacts directly with components of the nuclear pore complex and its import is inhibited by factors in cytosol (Fagotto *et al.*, 1998). This suggested a novel mode of nuclear import by interactions between the Arm repeats of ß-catenin and the nuclear pore machinery. ß-catenin can also be exported from the nucleus (Prieve and Waterman, 1999), and the authors speculate that this may be mediated by gsk3ß. A 19 amino acid deletion within Arm repeat 6 allows ß-catenin to become localised to the nucleus (Prieve and Waterman, 1999). As this region mediates binding to LEF/TCF and APC, these proteins are candidates for the previously proposed cytosolic factors (Fagotto *et al.*, 1998). Importantly, nuclear localisation of ß-catenin/LEF-1 complexes is not always sufficient for transcriptional activation. Nuclear ß-catenin/LEF-1 complexes can activate transcription of a reporter gene in transformed, but not wild type, T cells (Prieve and Waterman, 1999). Co-regulation of Wnt-dependent ß-catenin/TCF transcriptional activity can occur: a MAP kinase signalling cascade phosphorylates TCF during *C. elegans* development and in mammalian cells (Ishitani *et al.*, 1999; Meneghini *et al.*, 1999; Rocheleau *et al.*, 1999)

Nuclear regulation of transcriptional activation by ß-catenin and TCF may therefore be dependent on the cellular context. Indeed, Arm recruits a zinc finger protein, Teashirt (Tsh), into the nucleus for a subset of Wg signalling functions (Gallet *et al.*, 1999; Gallet *et al.*, 1998). Whether dTCF also complexes with Tsh is not known.

Interestingly, Tsh also complexes with zw3 (Gallet *et al.*, 1999). LEF-1 can work with coactivators other than ß-catenin to regulate non-Wg-responsive genes (Hsu *et al.*, 1998). dTCF acts with a corepressor, Groucho, to inhibit transcription of Wg-responsive genes in the absence of Arm (Levanon *et al.*, 1998). Thus, high levels of Arm may overcome repression by competing for binding sites on dTCF with Groucho. The importance of cellular context on transcriptional outcome was further demonstrated by the identification of *in vivo* target genes.

1.2.8 Target genes of ß-catenin/TCF signalling

Although genetic experiments had shown that transcription of genes such as *engrailed* in Drosophila (Siegfried *et al.*, 1992) and *gsc* in zebrafish (Kelly *et al.*, 1995) was modulated by Wg signalling, direct interaction of ß-catenin/TCF with promoters has only been demonstrated more recently. A known Wg target gene *in vivo*, the *Ubx* homeobox gene, contains a Wg response element in the midgut enhancer region which binds to Arm in a complex with coexpressed murine LEF-1 (Riese *et al.*, 1997). Transcriptional stimulation of *Ubx-lacZ* expression by mLEF-1 required Arm *in vivo*, and was also dependent on a neighbouring response element in the Ubx promoter activated by a Wg-independent signal (Riese *et al.*, 1997). Thus in an *in vivo* context, an active Arm/TCF complex was shown to be insufficient to activate a target gene unless the cells also received an additional activatory signal, presumably allowing another protein to bind the promoter and modulate the DNA-bending properties of TCF.

LEF-1/ß-catenin binds to an *E-cadherin* promoter fragment, which contains a consensus LEF/TCF binding site (Huber *et al.*, 1996). As *E-cadherin* -/- embryonic stem cells have high levels of LEF-1, the authors proposed that ß-catenin/LEF-1 acted to repress *E-cadherin* transcription. However, data from *Drosophila* suggested that increases in Arm via Wg signalling activate *DE-cadherin* transcription in cell culture (Yanagawa *et al.*, 1997), although the physiological relevance of this in the embryo (where levels of DE-cadherin are much higher than in cultured cells) remains questionable. In addition there may be genuine differences in the regulation of E-cadherin transcription between these different systems.

In *Xenopus*, the promoter of the dorsal organiser-specific gene *siamois*, a target of the ß-catenin-mediated dorsalisation pathway (Carnac *et al.*, 1996), is an *in vivo* target for ß-catenin/XTCF3 binding (Brannon *et al.*, 1997). A *siamois* reporter gene is activated to a much greater extent when injected dorsally (where ß-catenin levels are high) than ventrally, as would be expected. However, deletion of the XTCF3 binding

sites from the reporter construct increased its ability to be expressed ventrally, implying that XTCF3 has a role as a repressor of *siamois* transcription in the absence of ß-catenin (Brannon *et al.*, 1997).

Only three other direct gene targets of ß-catenin/TCF signalling are known (as defined by binding of ß-catenin/TCF to their promoter). These are *c-jun*, *fra-1* and *cyclin D1* (Mann *et al.*, 1999; Tetsu and McCormick, 1999), and all are upregulated in ß-catenin-dependent manner in colon carcinoma cells, emphasising that excess cellular ß-catenin can contribute to oncogenic growth by affecting cell proliferation. Both *c-myc* in colon carcinoma cells (He *et al.*, 1998) and *Dfz2* in *Drosophila* (Cadigan *et al.*, 1998) have been shown to be targets of Wg signalling; it would seem plausible that they could also be directly regulated by ß-catenin/TCF but as yet this is not proven.

1.2.9 Conservation of Wnt signalling

Figure 1.1 provides the current view of ß-catenin-mediated Wnt signalling in *Drosophila* and vertebrates, with interactions between the molecules involved. In *C. elegans* Wnt signals, their receptors, gsk-3ß, ß-catenin homologues, APC and LEF-1 type transcription factors have all been characterised. However, *C. elegans* gsk3ß appears to be activated by a Wnt signal (Schlesinger *et al.*, 1999) and *C. elegans* may not have an Axin homologue (Ruvkun and Hobert, 1998). In conclusion, Wnt pathway proteins are components a versatile developmental signalling "cassette" which is used many times throughout embryogenesis in metazoan systems to specify cell fates.

One important unanswered question is whether a Wnt signal promotes ß-catenin-mediated dorsal axis specification very early in *Xenopus* development. ß-catenin establishes an early "dorsal" signal over the entire animal-vegetal axis (Wylie *et al.*, 1996). Maternal ß-catenin and Xdsh are dorsally localised shortly after fertilisation, due to movement away from the vegetal pole during cortical rotation (Larabell *et al.*, 1997; Miller *et al.*, 1999; Rowning *et al.*, 1997). This is dependent on the microtubule cytoskeleton and is likely to be independent of a Wnt signal as no endogenous candidate Wnt has been found in the correct place in the very early embryo. *XWnt8b* (capable of axis induction and thought to specify dorsal fates) is maternally expressed, but in the animal hemisphere(Cui *et al.*, 1995). Perhaps XWnt8b acts to stabilise dorsally localised ß-catenin later in development.

Similarly in sea urchins, the nuclear localisation of ß-catenin in vegetal cells does not require a signal from the micromere cells. The authors suggest that ß-catenin could be localised to the nucleus in a cell-autonomous manner, independently of a Wnt

signal, as nuclear ß-catenin staining is seen in dissociated cells in similar proportions to in whole embryos. However nuclear ß-catenin is required for micromere cells to signal: micromeres without nuclear ß-catenin cannot induce a secondary body axis when transplanted into animal cells (Logan *et al.*, 1999). The association of certain Wnt signalling components with the cytoskeleton is interesting as ß-catenin is known to have a variety of cytoskeletal roles, as discussed in the next section.

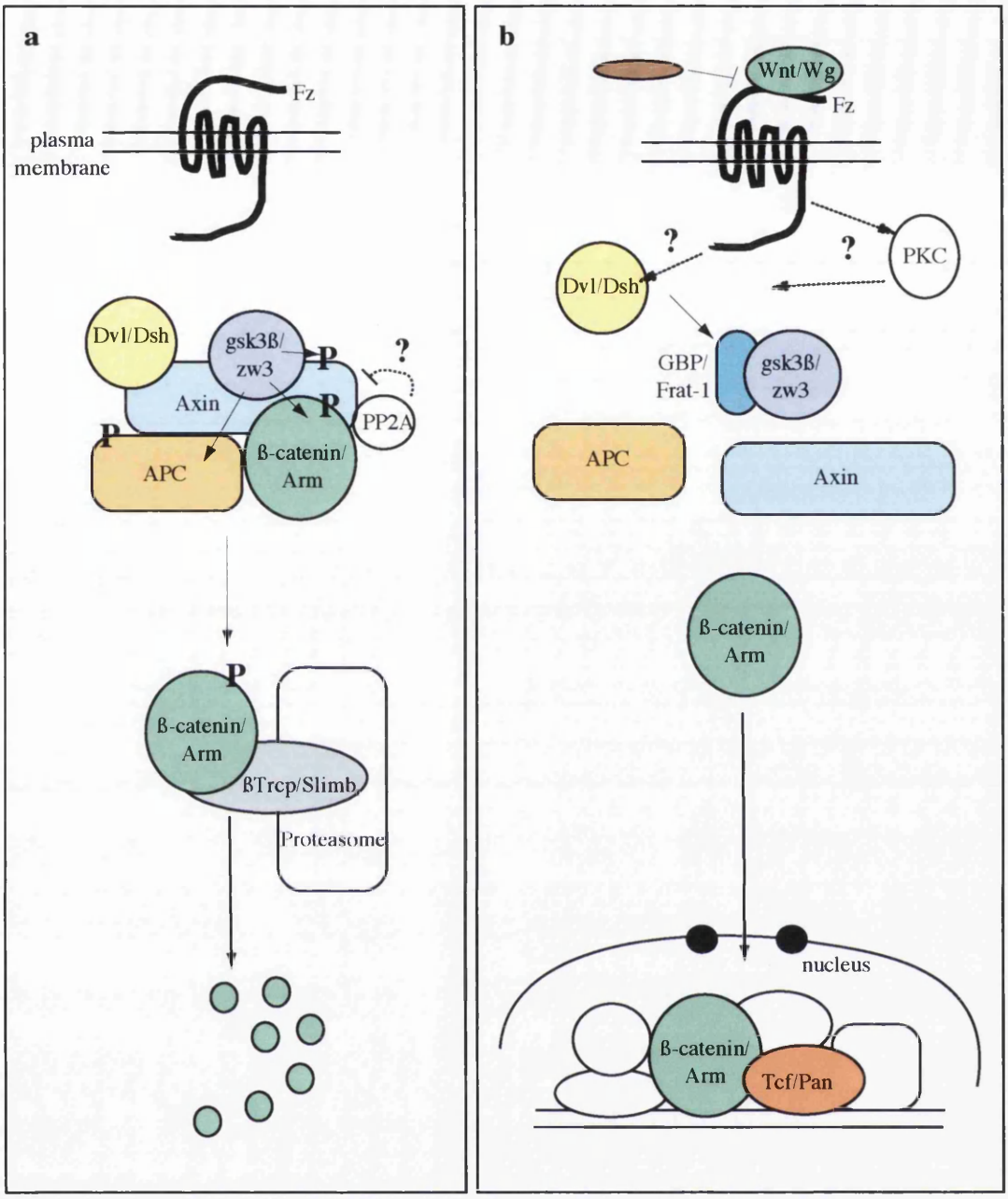

Figure 1.1 Overview of Wnt/Wg signalling

a) In the absence of Wnt/Wg signal, cytoplasmic Axin forms a scaffold on which a stable complex containing gsk3ß/zw3, ß-catenin/Arm, APC and Dvl/Dsh can form. gsk3ß/zw3 phosphorylates Axin promoting its stabilisation, and also phosphorylates ß-catenin and APC (**P**). Protein phosphatase 2A (PP2A) may negatively regulate this phosphorylation. Phosphorylated ß-catenin is recognised by ß-Trcp/Slimb and targeted to ubiquitin-mediated proteasomal degradation.

b) Wnt/Wg signal, binds to the Fz receptor. This binding can be antagonised by extracellular inhibitors (brown). Activation of fz transduces a signal to the cytoplasm via an unknown mechanism involving PKC. Dvl/Dsh to recruits an inhibitor of gsk3ß, GBP/Frat-1, to the complex. In the absence of phosphorylated Axin the complex is unstable and dissociates. The resulting stable, unphosphorylated ß-catenin can enter the nucleus via the nuclear pore (black circles) and complex with TCF/Pan to regulate gene transcription in conjunction with a variety of other proteins.

1.3 Cytoskeletal roles of Arm/ß-catenin

Cells of a multicellular organism must adhere together in an organised manner, concomitant with their differentiation, to form tissues. This process was long hypothesised to involve differential cell adhesion. Disaggregated animal cells from different tissues or germ layers can sort out when mixed (Townes and Holtfreter, 1955), as indeed can *Dictyostelium* cells of different species or cell type (Bozzaro and Ponte, 1995; Sternfeld, 1979). The generation of antibodies which block cell-cell adhesion in animals and *Dictyostelium* have shown that adhesion is mediated by specific cell surface molecules, rather than being a general property of the cell membrane as a whole (reviewed in (Bozzaro and Ponte, 1995; Stevenson and Paul, 1989)). The cadherin superfamily of molecules is essential for cell-cell adhesion in animals. Cadherin-based adhesion is dependent on calcium and cadherins are resistant to proteolysis in the presence of calcium (Kemler *et al.*, 1989). Fibroblasts which do not express cadherins acquire calcium-dependent adhesiveness when transfected with full length E-cadherin cDNA (Nagafuchi *et al.*, 1987). Cadherins such as E-cadherin are homophilic adhesion molecules (Nose *et al.*, 1990) and differential adhesion between cell types can be brought about by differences in the type or number of cadherins expressed by cells (Nose *et al.*, 1988; Nose *et al.*, 1990; Steinberg and Takeichi, 1994). Mouse embryonic stem cells lacking E-cadherin are non-adhesive and cannot contribute to wild type embryos (Larue *et al.*, 1996). Addition of wild type E-cadherin to these cells causes a dose-dependent reaggregation and allows epithelial formation. If N-cadherin is expressed in the place of E-cadherin the cells still adhere but from neural tissue instead of epithelium, demonstrating a role for cadherins in defining tissue identity (Larue *et al.*, 1996).

In some tissues such as epithelia, specialised areas of the plasma membrane which mediate cell-cell interactions can be identified by their morphology in the electron microscope (Stevenson and Paul, 1989). These intracellular junctions fall into four subgroups: adherens junctions, associated with the actin cytoskeleton; desmosomes, connected to intermediate (keratin) filaments; tight junctions, associated with actin, which restrict the movement of molecules between cell membranes by forming an impermeable barrier; and gap junctions which contain channels allowing small molecules and ions to pass from one cell to another. Classical cadherins such as E-cadherin are associated with adherens junctions and desmosomes contain more divergent desmosomal cadherins (Magee and Buxton, 1991). Assembly of all four

types of junctions is co-ordinately regulated by early cadherin-mediated cell contacts (Gumbiner and McCrea, 1993); antibodies to E-cadherin block the formation of adherens junctions, desmosomes and tight junctions in the presence of calcium in epithelial cells (Gumbiner *et al.*, 1988). If junctional assembly is inhibited in this manner or in the absence of calcium, the stability of junctional components is reduced and they are degraded within the cell (reviewed in (Magee and Buxton, 1991; Stevenson and Paul, 1989)).

Classical cadherins are linked to the actin cytoskeleton and act to generate tension at adhesion sites. The linkage is mediated by three cytoplasmic proteins which bind tightly to the C-terminus of E-cadherin (Ozawa *et al.*, 1989) and were termed α, ß and γ-catenins (from Latin catena, meaning chain). They form a detergent-insoluble complex with E-cadherin transfected into murine, human and avian cell lines and only elute from the complex at low pH. The affinity of γ-catenin for the complex is weaker than that of α or ß. A mutant E-cadherin lacking 37 amino acids of the C-terminal intracellular domain, which had previously been shown to be unable to mediate cell-cell adhesion and interaction with the cytoskeleton (Nagafuchi and Takeichi, 1988), cannot bind to the catenins (Ozawa *et al.*, 1989) or interact with F-actin (Ozawa *et al.*, 1990). α-catenin and actin bind to ZO1, a tight junctional component (Itoh *et al.*, 1997) and catenins recruit ZO1 to the membrane (Rajasekaran *et al.*, 1996), suggesting a molecular mechanism for the role of E-cadherin-containing intercellular contacts in the organisation of tight junctions.

The cytoplasmic region of E-cadherin is sufficient, as well as necessary, for catenin binding: a 72 amino acid segment of the cytoplasmic tail of E-cadherin coimmunoprecipitates the catenins when fused to a heterologous transmembrane protein (Ozawa *et al.*, 1990). These studies were extended to show that a neural cadherin (N-cadherin) could also associate with the three catenins (Ozawa and Kemler, 1992). Binding studies with recombinant proteins show that ß-catenin interacts directly with the cadherin cytoplasmic tail with a 1:1 stochiometry and promotes the assembly of the cadherin/ß-catenin/α-catenin complex (Aberle *et al.*, 1994). Pulse-chase and cross-linking analysis reveal that ß-catenin associates with a newly synthesised cadherin precursor *in vivo* and α-catenin is added later (Ozawa and Kemler, 1992). α-catenin can be selectively removed from the complex by octyl glucoside whilst leaving the ß-catenin/cadherin interaction intact. α-catenin links the complex to actin filaments and has homology to vinculin, a protein associated with actin at cell-substratum contacts (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991; Rimm *et al.*, 1995).

Cadherin-catenin-based adhesion systems are necessary for the dynamic rearrangement of cells during morphogenesis. These processes require the generation of forces between cells via the actin cytoskeleton to bring about cell migration and changes in cell shape. Arm is part of a membrane-associated complex with *Drosophila* α-catenin, *Drosophila* E-cadherin and actin (Oda *et al.*, 1994; Oda *et al.*, 1993; Peifer, 1993; Peifer *et al.*, 1991; Peifer and Wieschaus, 1990; Riggleman *et al.*, 1990). Electron microscopic studies of the developing gut epithelium, a tube of single cell thickness enclosing the gut lumen, revealed apicolateral spot-like adhesive junctions connecting the cells which contained *D*α-catenin, Arm and a cadherin-like molecule (Peifer, 1993). High levels of *D*E-cadherin are required in tissues where cells move dynamically. Maternal *D*E-cadherin is sufficient for the formation of static epithelia. However, removal of zygotic *D*E-cadherin impairs morphogenetic movements during oogenesis, formation of the midgut epithelium, convergent-extension and delamination in the neurectoderm and outgrowth and branching of the Malpighian tubules (Cox *et al.*, 1996; Tepass *et al.*, 1996; Uemura *et al.*, 1996). Elimination of *arm* from the germline results in a loss of junctions and cell-cell adhesion within the follicle cells, loss of adhesion between the germ cells (which contain diffuse cell-cell contacts rather than junctions) and loss of the heterologous germ cell-follicle cell contacts (Peifer *et al.*, 1993). Loss of Arm disrupts germ cell shape and actin organisation leading to aberrant nuclear positioning and detachment of actin ring canals from the membrane (Peifer *et al.*, 1993). *D*E-cadherin is essential for germ cell rearrangements that localise the oocyte to the posterior of the egg chamber. A heterophilic interaction between the *D*E-cadherin-expressing oocyte and follicle cells occurs, with the posterior follicle cells expressing more *D*E-cadherin than other follicle cell types (Gonzalez-Reyes and St Johnston, 1998). In addition, *D*E-cadherin is essential for the migration of follicle cells over the germ cells during oogenesis: the speed of migration of a patch of follicle cells, which moves in a co-ordinated manner, is reduced when levels of *D*E-cadherin are lowered (Niewiadomska *et al.*, 1999).

Junctions containing cadherin, α-catenin and ß-catenin-like proteins localise to juxtaposing cells in the four-cell *C. elegans* embryo, but are not required for general static intracellular adhesion (Costa *et al.*, 1998). The proteins are present in junctions in the hypodermal epithelium, an initially flat sheet of cells which spreads over the surface of the embryo to eventually surround it. Cells at the leading edge of the hypodermis crawl over the embryo and meet on the ventral surface. Closure of the hypodermis generates forces that squeeze the embryo into a long, thin worm. Worms mutant for

cadherin, α-catenin or ß-catenin cannot complete hypodermal spreading and closure, including an actin purse-string contraction to close the posterior end of the hypodermal tube (Costa *et al.*, 1998). All these processes require the generation of actin tension. A similar purse-string contraction mechanism operates at the exposed leading edges of cells in wounded epithelia, where a ring of actin forms around the wound, to pull cells together during wound closure. The continuity of the actin ring between cells is mediated by E-cadherin/α-catenin/ß-catenin, which anchor actin filaments to the membrane. Antibodies to E-cadherin block actin filament assembly and wound closure (Danjo and Gipson, 1998). The contractility of the ring may be mediated by myosin II which colocalises with the actin filaments (Danjo and Gipson, 1998). Similarly to *C. elegans* hypodermal mutants, moderate *arm* alleles, which produce sufficient Arm protein to participate in embryonic signalling, cannot form a single-layered epithelium enclosing the embryo, resulting in a block to gastrulation and dorsal closure (Cox *et al.*, 1996). Presumptive epithelial cells round up, become apolar and cannot adhere to one another or form adherens junctions. Instead, the epithelial cells acquire a mesenchymal appearance and do not form adherens junctions (Cox *et al.*, 1996; Muller and Wieschaus, 1996). A neural splice variant of Arm, lacking the C-terminus, shapes axon fascicles via cell-cell adhesion in the central nervous system, probably in conjunction with *D*N-cadherin (Loureiro and Peifer, 1999).

ß-catenin knock-out mice make no mesoderm (Haegel *et al.*, 1995) and have an adhesion defect in the ectoderm. Tumour cell lines lacking wild type catenins are non-adhesive but their transformed phenotype can be reversed by the expression of the cognate wild type gene (Hirano *et al.*, 1992). Moreover, an ovarian cancer cell line containing mutant α-catenin, lacking the ß-catenin binding site, has its transformed phenotype rescued by expression of wild type α-catenin: deregulated growth is retarded and the cells gain an adhesive, epithelial morphology (Bullions *et al.*, 1997). Maternal depletion of α-catenin in *Xenopus* embryos leads to a loss of adhesion in the blastula, similar to that seen by depleting EP-cadherin (Kofron *et al.*, 1997). A fragment of *Xenopus* C-cadherin lacking the extracellular domain, which acts in a dominant negative fashion inhibiting catenin binding to endogenous E-cadherin, globally disrupts calcium-dependent intracellular adhesion and epithelial formation (Kintner, 1992).

1.4 Are the signalling and cytoskeletal functions of Armadillo/ß-catenin independent?

Given that both intracellular signal transduction and regulated cell-cell adhesion are fundamental to development, are the functions of Arm/ß-catenin in these two processes coupled? An *in vivo* structure-function study of *arm* mutants hinted at the independence of the signalling and structural roles of Arm (Orsulic and Peifer, 1996b). Various *arm* deletion constructs were tested for their ability to complement *wg* or *arm* signalling defects and their intracellular location and *in vitro* binding properties were assayed. An amino terminal region of Arm binds *D*α-catenin (Orsulic and Peifer, 1996b; Pai *et al.*, 1996), in agreement with the result obtained with mammalian proteins (Aberle *et al.*, 1996). Mutants with this region deleted are still functional in Wg signalling. Arm repeats 3-8, which bind cadherin *in vitro* (Pai *et al.*, 1996) are essential for both adhesive function and Wg signalling, although additional regions are also required for signalling (Orsulic and Peifer, 1996b). Importantly, introducing a mutant *arm* that cannot signal, and a mutant *arm* that cannot go to junctions, into an *arm* background, produces a wild type fly (Orsulic and Peifer, 1996b). Thus, the two functions of Arm can be fulfilled by two discrete proteins within the embryo. This idea was explored further in a set of elegant experiments in the *Drosophila* imaginal discs and wing. Targeted overexpression of either full-length, functional, or truncated, non-adhesive, *D*E-cadherin mimics the *arm* patterning phenotype (as assayed by morphology and *engrailed* expression), even though the cadherin constructs have opposing effects on adhesion (Sanson *et al.*, 1996). This shows that the "signalling" pool of Arm can be titrated by binding to the cadherin tail, and suggests that the primary function of the Wg signal is not to affect cell adhesion. It is interesting to note that, of the three *C. elegans* ß-catenin homologues, one has a purely adhesive role, as mutants undergo normal differentiation (Costa *et al.*, 1998), while the other two have thus far only been shown to affect cell signalling (Eisenmann *et al.*, 1998; Maloof *et al.*, 1999; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997).

Studies in *Xenopus* (Heasman *et al.*, 1994) have shown that ß-catenin signalling during embryogenesis can be disrupted without perturbing adhesion, although residual junctional ß-catenin was present in these experiments (Kofron *et al.*, 1997). Similarly, antibodies that block ß-catenin signalling in *Xenopus* do not perturb cell-cell adhesion (McCrea *et al.*, 1993). Deletion mutants of ß-catenin, without the α-catenin binding domain, are necessary and sufficient for axis duplication (Fagotto *et al.*, 1996; Funayama *et al.*, 1995). At the biochemical level, the binding of ß-catenin to E-

cadherin is mutually exclusive with its binding to APC (Hulsken *et al.*, 1994; Rubinfeld *et al.*, 1995; Shibata *et al.*, 1994). Thus *in vivo*, the cellular function of Arm/ß-catenin may be tightly regulated by the level of its available binding partners. Indeed, addition of nuclear localisation or nuclear export signals to Arm has no effect on its *in vivo* function or regulation when an Arm transgene is expressed at near-physiological levels (Cox *et al.*, 1999).

Cell fractionation has identified many intracellular "pools" of ß-catenin, which can be separated biochemically, and include junctional and monomeric ß-catenin in addition to unidentified ß-catenin complexes (Hinck *et al.*, 1994a; Nathke *et al.*, 1994; Stewart and Nelson, 1997). Any signal or artificial manipulation that alters the distribution of ß-catenin between these pools could lead to an effect on signalling or adhesion. Many experiments have shown that manipulations that lead to perturbation of endogenous ß-catenin level or location can in turn affect signalling. In systems such as *Xenopus*, many experiments involve high-level overexpression of the protein of interest that may saturate its normal binding partners. In *Xenopus* and sea urchins, as in *Drosophila*, blocking ß-catenin signalling can be achieved by overexpression of full length C-cadherin or its intracellular domain (Heasman *et al.*, 1994; Logan *et al.*, 1999; Wikramanayake *et al.*, 1998). The axis-inducing ability of a mutant ßTrcp is also suppressed by coexpression of C-cadherin (Marikawa and Elinson, 1998). Reduction of the levels of *D*E-cadherin in *Drosophila* embryos can suppress the segment polarity phenotype (Cox *et al.*, 1996), and the cytoplasmic tail of N-cadherin can bind nuclear ß-catenin and compete it away from LEF-1, inhibiting transcription of a reporter gene in mammalian cells (Sadot *et al.*, 1998).

Thus although cadherins can affect the developmental roles of Arm/ß-catenin, this is likely to be mediated by indirect titration effects on endogenous "signalling pools" of ß-catenin/Arm. However, Wnt-1 can control the adhesive properties of cultured cells by stabilising cell-cell contacts and modulating gap junctional communication (Bradley *et al.*, 1993; Hinck *et al.*, 1994c; Olson *et al.*, 1991). In addition, *E-cadherin* is a target gene of Wnt signalling, as discussed in section 1.2. Convincing *in vivo* evidence for a link between Wnt signalling and components of the cytoskeleton has come from cell biological studies. Sensitive confocal microscopic analysis (Larabell *et al.*, 1997) revealed dorsoventral asymmetry of endogenous ß-catenin in the peripheral cytoplasm of two-cell *Xenopus* embryos, with highest levels of ß-catenin on the future dorsal side. Elegant studies of *Xenopus* eggs showed that cortical rotation, which occurs after fertilisation, causes Xdsh to be transported along a cortical

array of parallel microtubules. This localises Xdsh to presumptive dorsal areas of the single-celled egg, thus creating asymmetry before the first cleavage occurs (Miller *et al.*, 1999). ß-catenin localises to dorsal, but not ventral, microtubules (Rowning *et al.*, 1997), presumably stabilised by Xdsh. This microtubule-mediated localisation is necessary for formation of the embryonic axis, as the asymmetric localisation of Xdsh is randomised by D_2O, which disrupts microtubule organisation and dorsalises embryos. Movement of Xdsh is abolished by UV treatment, which ventralises embryos (Miller *et al.*, 1999). It is interesting to note that APC localises to the growing end of microtubules (Nathke *et al.*, 1997) and interacts with EB1, a microtubule-binding protein (Berrueta *et al.*, 1998), as well as affecting ß-catenin stability (Munemitsu *et al.*, 1995) and causing axis duplication (Vleminckx *et al.*, 1997). Zygotic *Drosophila* APC is expressed in axon tracts and motor neurones in the central nervous system. Zygotic *dAPC* mutant embryos have defects in the fasciculation of the axonal scaffold (Hayashi *et al.*, 1997), similar to those seen in neural *arm* mutants (Loureiro and Peifer, 1999), suggesting a cytoskeletal role for dAPC. In developing mammalian neurones, a Wnt signal induces axonal remodelling by inhibiting gsk3ß-dependent phosphorylation of a microtubule associated protein, MAP-1B (Lucas *et al.*, 1998). Whether this process also involves APC or ß-catenin, the levels of which do increase in neurones where gsk3ß is inhibited (Lucas and Salinas, 1997), is not known. Phosphorylation of another microtubule-binding protein, tau, by gsk3ß can be inhibited by Dvl-1 overexpression (Wagner *et al.*, 1997).

Wnt signals also affect the cytoskeleton independently of their effects on ß-catenin. In *C. elegans*, some components of the Wnt signalling system required for gut induction directly regulate the orientation of the mitotic spindle. This process requires the Fz receptor and gsk-3ß, but not ß-catenin, TCF and APC; in addition, redundant Wnt signals may operate (Rocheleau *et al.*, 1997; Schlesinger *et al.*, 1999; Thorpe *et al.*, 1997). Specification of tissue polarity in *Drosophila* involves *fz*, *dsh* and rho family GTPases, which regulate the actin cytoskeleton (Boutros *et al.*, 1998; Krasnow *et al.*, 1995; Strutt *et al.*, 1997; Theisen *et al.*, 1994), but does not require Arm. Interestingly, Fz tissue polarity signalling is perturbed by mutations in *dachsous*, a *Drosophila* cadherin-like gene (Adler *et al.*, 1998).

Adherens junctions are associated with a number of signalling molecules, which could provide a mechanism whereby components of signalling pathways can become localised together. Formation of adherens junctions recruits PI-3-kinase to the E-cadherin complex and leads to activation of PKB/akt (Pece *et al.*, 1999). Junctional

stability has been proposed to be regulated by tyrosine phosphorylation of cadherins, ß-catenin and other junctional components by src family kinases (Hiscox and Jiang, 1999; Kellie, 1988; Takeda *et al.*, 1995; Tsukita *et al.*, 1991). ß-catenin has been proposed to act as a regulatable linker in junctional assembly (Hinck *et al.*, 1994c; Nagafuchi *et al.*, 1994). ß-catenin associates with, and may be modulated by, EGF and VEGF receptor tyrosine kinases (Hazan and Norton, 1998; Hoschuetzky *et al.*, 1994; Ilan *et al.*, 1999) and protein tyrosine phosphatases (Muller *et al.*, 1999b). ß-catenin and gsk3ß can also be regulated by signals from cell-matrix contacts (Delcommenne *et al.*, 1998; Novak *et al.*, 1998), suggesting a co-ordinate regulation of the various adhesion systems within a cell.

In conclusion, as development is an integrated process, it seems intuitive that the signalling and adhesive functions of ß-catenin/Arm, although genetically separable in *Drosophila*, will be coregulated. However, dissecting this regulation in animal systems is complex and many experiments performed so far involve artificial overexpression of the molecules involved, leading to results that require careful interpretation. Using *Dictyostelium* to study the relationship between signalling and adhesion has the advantage that cell biological studies can be performed in a genetically tractable background.

1.5 Plakoglobin

ß-catenin and Armadillo also share high similarity with plakoglobin, a molecule enriched in desmosomes and also present in adherens junctions (Cowin *et al.*, 1986); (Franke *et al.*, 1989). ß-catenin and plakoglobin are distinct proteins (Peifer *et al.*, 1992); (Butz *et al.*, 1992). Antibodies to Arm cross-react with ß-catenin but not plakoglobin, and plakoglobin, but not ß-catenin, coprecipitates with desmosomal cadherins (Peifer *et al.*, 1992). Plakoglobin is identical to γ-catenin, and coimmunoprecipitates with both E- and N-cadherin. The binding of plakoglobin to cadherins is weaker than that of ß-catenin (Knudsen and Wheelock, 1992; Peifer *et al.*, 1992), as seen for γ-catenin (Ozawa *et al.*, 1989; Ozawa and Kemler, 1992). Plakoglobin colocalises with cadherins at sites of cell-cell contact; "patching" of N-cadherin with divalent antibodies in living cells results in a cognate redistribution of plakoglobin (Knudsen and Wheelock, 1992). ß-catenin is not found associated with desmosomal components. The molecular partners of plakoglobin in desmosomes are distinct from those in adherens junctions. Plakoglobin binds to the intracellular tail of desmosomal, as well as classical, cadherins and another molecule, desmoplakin, binds

to plakoglobin and links the membrane-associated complex to the intermediate filament network (Kouklis *et al.*, 1994; Kowalczyk *et al.*, 1997). Plakoglobin is essential for the clustering together of desmosomal cadherins in the plasma membrane, and is the key to promoting desmosomal assembly after initial E-cadherin-containing cell-cell contacts have formed (Kowalczyk *et al.*, 1997). Transfection of E-cadherin and plakoglobin into a non-adhesive cell line allows the formation of adherens junctions and desmosomes; with E-cadherin and ß-catenin only adherens junctions form (Lewis *et al.*, 1997). The mechanism by which plakoglobin co-ordinately regulates formation of the two junctional types is not clear: no molecular exchange of plakoglobin between the junctions need occur, as an E-cadherin-plakoglobin fusion protein can promote the assembly of both adherens junctions and desmosomes (Lewis *et al.*, 1997).

Plakoglobin-depleted *Xenopus* embryos are flattened in shape, and show mild adhesion defects and delayed gastrulation, but do not have defects in embryonic axis specification (Kofron *et al.*, 1997). These defects could be rescued by overexpression of *Xenopus* plakoglobin in a dose-dependent manner, but not by overexpression of ß-catenin (Kofron *et al.*, 1997). Plakoglobin is upregulated in ß-catenin knockout mice (Ruiz *et al.*, 1996), but appears not to compensate for ß-catenin in its signalling or adhesive roles. Plakoglobin knockout mice, on the other hand, have no embryonic patterning defects, but lack desmosomes and show a merging of adherens junction and desmosomal components, leading to skin blistering and lethal structural defects in their heart tissue (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996). No *Drosophila* homologue of plakoglobin has yet been reported, perhaps Arm can fulfil the adhesive role of both vertebrate proteins.

Plakoglobin has been proposed to be a tumour suppressor gene, and loss-of-heterozygosity of plakoglobin is observed in breast and ovarian tumours (Aberle *et al.*, 1995). The tumour suppressor effect may be independent of cell adhesion, as transfection of plakoglobin into a renal carcinoma cell line inhibits tumourigenicity of the cells in a dose-dependent manner in the absence of any junctional components (Simcha *et al.*, 1996). Alternatively, undetectable levels of junctional components may be present in these cells, as the tumour suppressor effect of plakoglobin is enhanced by cotransfection of N-cadherin (Simcha *et al.*, 1996). Formation of desmosomes by transfection of plakoglobin and desmosomal cadherins into a non-adhesive cell line inhibits the invasive phenotype of these cells (Tselepsis *et al.*, 1998).

Whether plakoglobin plays a signalling role during development remains controversial. Plakoglobin interacts *in vitro* with many partners of ß-catenin including

TCF, APC and Axin (Behrens *et al.*, 1996; Kodama *et al.*, 1999; Molenaar *et al.*, 1996; Rubinfeld *et al.*, 1995; Shibata *et al.*, 1994). Overexpression of full-length *Xenopus* plakoglobin induces axis duplication (Karnovsky and Klymkowsky, 1995; Rubenstein *et al.*, 1997). The exogenous protein accumulates in the nucleus (Rubenstein *et al.*, 1997), and as with ß-catenin and classical cadherins, both axis duplication and nuclear localisation are suppressed by coinjection of the desmoglein tail. An N-terminal truncation of plakoglobin, lacking the gsk3ß phosphorylation sites, has more potent axis inducing ability than the full-length form, as for ß-catenin (Kelly *et al.*, 1995; Rubenstein *et al.*, 1997; Yost *et al.*, 1996). However, overexpression of plakoglobin without the α-catenin binding site perturbed embryonic cell-cell adhesion, in addition to inducing an axis (Rubenstein *et al.*, 1997), presumably acting in a dominant-negative fashion and sequestering cadherin in a non-functional complex. Exogenous plakoglobin deletion constructs that could not induce ectopic axes also localised to the nucleus (Rubenstein *et al.*, 1997). These observations raised the question of how exogenously overexpressed molecules, or molecular fragments, were interfering with the normal interplay of ß-catenin/plakoglobin and their binding partners in the cell. Could all the "signalling" activities of plakoglobin be simply attributed to displacement of ß-catenin from the membrane, into pools where it could signal ectopically?

A membrane-anchored form of plakoglobin that cannot enter the nucleus causes axis duplication. No detectable effect is seen on the levels or distribution of endogenous ß-catenin (Merriam *et al.*, 1997). The authors suggest a model whereby membrane-tethered plakoglobin could be sequestering, for example, XTCF3 at the membrane and preventing it from repressing dorsal gene expression, hence causing dorsal axis duplication. However, this proposed signalling role of plakoglobin was questioned (Miller and Moon, 1997). A membrane-localised ß-catenin lacking the cadherin binding domain has its axis-inducing ability removed by coexpression of C-cadherin. This implies that the membrane-anchored ß-catenin relocates some endogenous ß-catenin to a "signalling" pool, where it is titrated back to the membrane by adding more C-cadherin to the cell. In addition, careful confocal analysis reveals changes, including nuclear localisation, in the distribution of endogenous ß-catenin, when plakoglobin was overexpressed in the embryo. Plakoglobin is not seen in the nucleus, but relocates APC protein (Miller and Moon, 1997). These data suggested exogenous plakoglobin and endogenous ß-catenin might compete for a component of the degradation machinery.

Mammalian ß-catenin and plakoglobin, expressed from the *arm* promoter in *Drosophila*, were introduced into *arm* mutant embryos (White *et al.*, 1998). Both molecules rescue the *arm* adhesion defect in the germline, and the adhesion defect of weak *arm* alleles in the embryo. In addition, both form a functional complex with *D*E-cadherin and *D*α-catenin *in vivo*. However, the segment polarity defect of strong zygotic *arm* alleles is only partly rescued by ß-catenin, and plakoglobin only rescues the *arm* phenotype if maternal *arm* is present. The plakoglobin-based segment polarity rescue seen is likely to be due to the titration of maternal Arm from the junctions into the cytosol, as plakoglobin is never seen in the cytosol in the striped pattern expected if it were regulated by Wg (White *et al.*, 1998). Since ß-catenin does not fully rescue the *arm* signalling defect cross-species differences in function appear to exist.

Experiments performed in mammalian cell lines have further highlighted the problems with overexpression studies. Even a low level of exogenously expressed plakoglobin can downregulate endogenous ß-catenin from cell junctions, in a dose-dependent manner (Salomon *et al.*, 1997). Indeed, five-fold overexpression of plakoglobin in epithelial cells causes endogenous ß-catenin to enter the nucleus and activate LEF-1-dependent transcription (Simcha *et al.*, 1998). In a similar manner, expression of N-terminally deleted ß-catenin reduces the levels of endogenous ß-catenin: the stable mutant protein competes for binding to N-cadherin, and displaces junctional ß-catenin into the cytosol, where it is rapidly degraded (Munemitsu *et al.*, 1995; Salomon *et al.*, 1997). Membrane-anchored ß-catenin can stimulate transcription from a LEF-1 reporter gene implying, but not demonstrating, that endogenous ß-catenin is being displaced from the membrane to the nucleus (Hsu *et al.*, 1998). Overexpression of ß-catenin or plakoglobin allows their accumulation in nuclear aggregates, but only ß-catenin recruits LEF-1 to the aggregates, suggesting functional differences between the two catenins. Overexpression of ß-catenin does not relocate endogenous plakoglobin, which is largely found in desmosomes and is inaccessible to ß-catenin (Simcha *et al.*, 1998).

Thus vertebrates have two highly related Armadillo-related proteins with distinct but overlapping functions in cell adhesion. Plakoglobin seems dispensable for early developmental signalling events in vertebrates, and has no signalling ability in *Drosophila*. Many experiments throw doubt on any positive signalling function of the observed plakoglobin/TCF complexes (Behrens *et al.*, 1996; Huber *et al.*, 1996), although none preclude the possibility that plakoglobin could sequester transcription factors in the cytoplasm, and hence regulate gene expression indirectly (Merriam *et al.*,

1997), as may also be the case for the wrm1/POP-1 interaction in *C. elegans* (Han, 1997). All the data presented so far suggests that any perceived transcriptional properties of plakoglobin could be due to a titration effect on endogenous ß–catenin.

1.6 The armadillo protein superfamily

Cloning of more divergent Armadillo-related genes reveals an emerging superfamily of functionally distinct proteins, present in all eukaryotes. These proteins, defined by containing a tandem array of degenerate ~42 amino acid repeats, have a diverse range of functions in unicellular and multicellular organisms. Many of these proteins function in adhesion, and colocalise to adherens junctions and desmosomes, where they perhaps modulate the stability or strength of these junctions. p120ctn (with ten Arm repeats) is a tyrosine kinase substrate found at E-cadherin/ß-catenin-containing adherens junctions (Aghib and McCrea, 1995; Daniel and Reynolds, 1995; Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995). p120 binds a juxtamembrane region of E-cadherin, distinct from the ß-catenin binding site, and promotes lateral clustering of cadherin molecules (Yap *et al.*, 1998). δ-catenin, a brain-specific relative of p120, also interacts with the p120 juxtamembrane region, and confers a migratory phenotype on epithelial cells, increasing their scattering in response to hepatocyte growth factor (Lu *et al.*, 1999). Other members of the p120 subfamily, the plakophilins and p0071, have adhesive roles, particularly in desmosomes (Bonne *et al.*, 1999; Hatzfeld *et al.*, 1994; Hatzfeld and Nachtsheim, 1996). Plakophilin-1 binds to the N-terminus of desmoplakin and strengthens the interaction between desmoplakin/plakoglobin and desmosomal cadherins (Kowalczyk *et al.*, 1999). p120 and plakophilin 3 are found in the nuclei of cells (Bonne *et al.*, 1999; van Hengel *et al.*, 1999). p120 can also bind to a zinc-finger-containing transcription factor (Daniel and Reynolds, 1999). Whether these Armadillo proteins can function to directly regulate gene expression *in vivo* remains an open question.

A ß-catenin-like protein exists in a unicellular eukaryote, *Saccharomyces cerevisiae*. Vac8p (YEB3/Yel013p) contains eleven repeats, most similar in sequence to those of Arm/ß-catenin and plakoglobin (Fleckenstein *et al.*, 1998; Pan and Goldfarb, 1998; Wang *et al.*, 1998). Importantly, *Schizosaccharomyces pombe* has an uncharacterised gene homologous to *vac8*. Vac8p is necessary for vacuole integrity and inheritance, as *vac8* mutants cannot target new vacuolar membrane to the bud site during cell division. Vac8p is associated with the vacuolar membrane and cosediments with filamentous actin (Pan and Goldfarb, 1998; Wang *et al.*, 1998). Vac8p also

regulates import of proteins from the cytoplasm to the vacuole (Fleckenstein *et al.*, 1998; Wang *et al.*, 1998).

Another subfamily of Arm proteins has been identified, which may have a dual signalling and cytoskeletal role. Guanine nucleotide exchange factors, smgGDS, with eleven Arm repeats, have been identified in animals and *Dictyostelium* (Peifer *et al.*, 1994a; Vithalani *et al.*, 1998). Some of these proteins bind to yet other Armadillo repeat proteins, which in turn regulate kinesin ATPase motors involved in vesicular transport along microtubules, in animal systems (Shimizu *et al.*, 1996; Wedaman *et al.*, 1996). The APC protein, also required for signalling and adhesion as discussed previously, contains seven Arm repeats, the function of which is not known. No role has been demonstrated for the repeats in Wg/Wnt signalling or microtubule binding. Other more divergent Arm repeats have been identified in the regulatory subunit of protein phosphatase 2A and in ß-adaptin, a component of endocytic coated pits (Kussel and Frasch, 1995).

A subfamily of Arm repeat containing proteins mediates the nuclear import of proteins with nuclear localisation signals. These importin-α/karyopherin-α proteins have been cloned in yeast, mammals and *Drosophila* and contain ten repeats (Conti *et al.*, 1998; Kussel and Frasch, 1995; Sweet and Gerace, 1995; Torok *et al.*, 1995; Yano *et al.*, 1994). Importin-α interacts with the nuclear pore complex of proteins via importin-ß (Sweet and Gerace, 1995). Yeast importin-α is also required for maintaining nuclear structure, gene transcription and microtubule regulation (Yano *et al.*, 1994). Whether these functions are dependent on a role in nuclear import is unclear; perhaps this one protein in yeast fulfils the role of multiple diverged proteins in higher organisms. Interestingly, importin-ß is largely comprised of divergent tandem repeats known as HEAT motifs, of which Arm repeats are a subgroup (Andrade and Bork, 1995; Malik *et al.*, 1997). It is known that ß-catenin can also interact with the nuclear pore complex, via its Arm repeats, in addition to its junctional and signalling roles (Fagotto *et al.*, 1998). HEAT motifs have been found in eukaryotic and prokaryotic proteins, showing that proteins with this structure arose very early in evolution.

At first glance, proteins of the Armadillo superfamily seem to have very divergent functions within the cell. It is thought that the tertiary structure of these proteins, a right handed superhelix formed by the repeats, allows them to act as molecular adaptors on which to assemble specific protein complexes (Conti *et al.*, 1998; Huber *et al.*, 1997a; Peifer *et al.*, 1994a). However, broadly speaking, Arm family proteins mediate interactions between a membrane (either an intracellular membrane or

the plasma membrane) and a component of the cytoarchitecture. These interactions often require other linker proteins, and in some cases lead to protein translocation across membranes. A "primeval" protein with HEAT/Arm-like repeats may have existed in an ancestral cell, and regulated cell shape, motility or protein transport. Tandem repeats could be duplicated or lost and could undergo sequence divergence throughout evolution, to create a multifunctional family of proteins (figure 1.2). This divergence may have occurred prior to the evolution of multicellular organisms, as yeasts have two Arm-related proteins. When did the developmental Wg/Wnt signalling function of Armadillo proteins arise? Arm/ß-catenin signalling may be unique to metazoa, as current literature suggests. Figure 1.2 illustrates the variety of metazoa possessing one or more ß-catenin homologue with a developmental role. Many of these are known to be regulated by gsk3ß (section 1.2), or at least a lithium-sensitive activity (Yoshida *et al.*, 1998). ß-catenin and plakoglobin are found in vertebrates: *Xenopus*, mouse, human, zebrafish, and avian, canine and bovine systems (Butz *et al.*, 1992; Fouquet *et al.*, 1992; Franke *et al.*, 1989; Kelly *et al.*, 1995; Knudsen and Wheelock, 1992; McCrea *et al.*, 1991; Nollet *et al.*, 1996). At least one ß-catenin homologue is present in sea urchins (Miller and McClay, 1997), Ascidians (Yoshida *et al.*, 1998), and *Hydra* (Hobmayer *et al.*, 1996). *C. elegans* has three ß-catenin-like proteins (Costa *et al.*, 1998; Eisenmann *et al.*, 1998; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997).

However, plant sequences related to ß-catenin and vac8p exist in EST databases, the function of which is unknown. Plants also have gsk3ß-like kinases (Bianchi *et al.*, 1994; Pay *et al.*, 1993). *Dictyostelium* has a homologue of gsk3ß (gskA) with a developmental function (Harwood *et al.*, 1995). Did the signalling role of Arm/ß-catenin arise concomitantly with the advent of multicellularity, before the evolution of metazoa? Could this role be fundamental to the generation of a multicellular organism? If so, a *Dictyostelium* Arm-like protein would be predicted to participate in a developmental signalling pathway in a manner analogous to ß-catenin/Arm, and is also likely to function in intracellular adhesion.

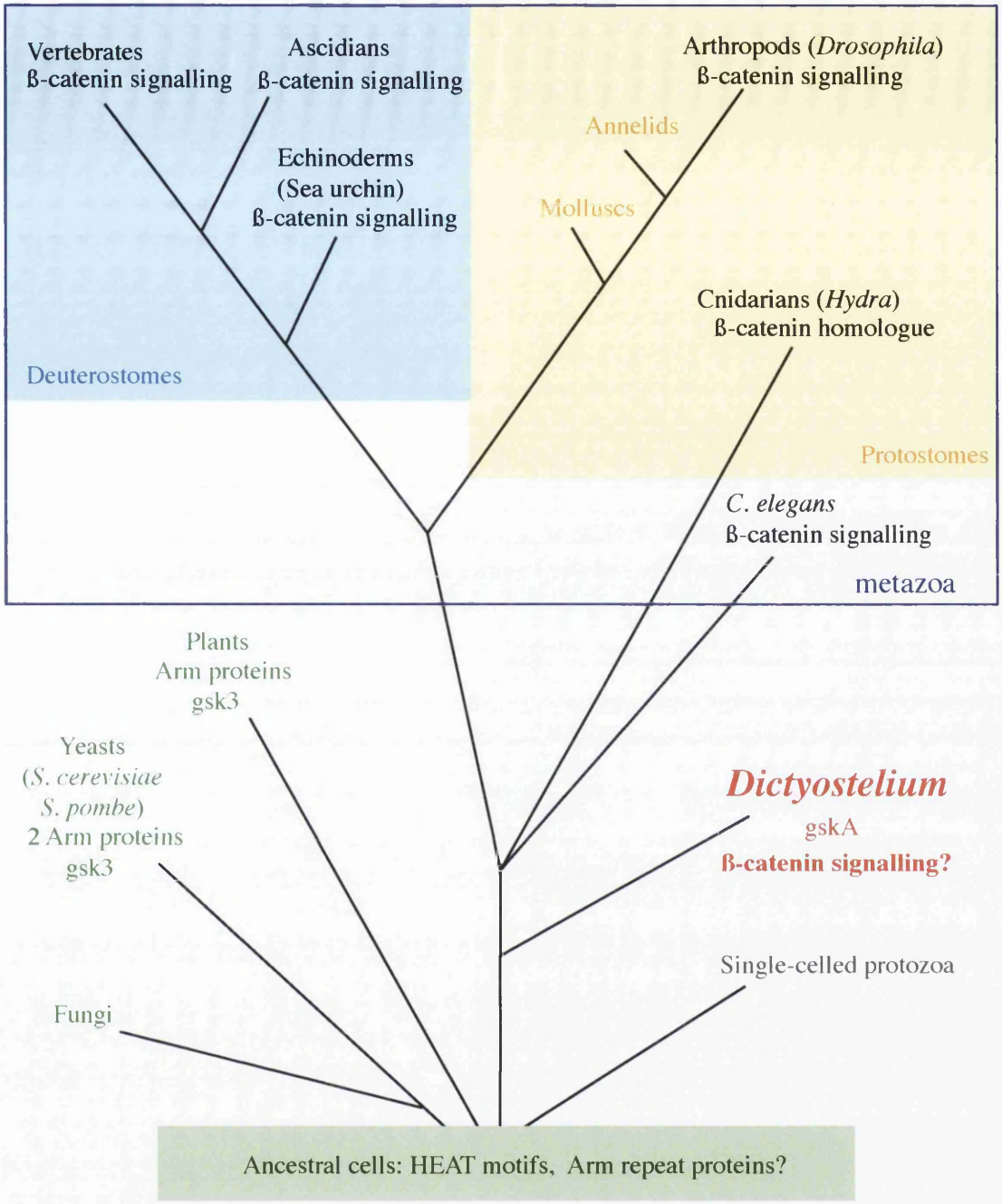

Figure 1.2 When did the signalling function of ß-catenin arise during evolution? Arm repeat proteins probably arose very early in evolution, in "ancestral" cells. Developmental ß-catenin signalling has been characterised in most phyla of metazoa. Non-metazoan multicellular organisms such as plants and *Dictyostelium* also contain Arm repeat proteins and a potential regulator of ß-catenin signalling, gsk3/gskA, but whether a developmental ß-catenin signalling pathway analogous to that in metazoa exists in these organisms is unknown. One aim of my work is to identify a non-metazoan developmental ß-catenin signalling pathway in *Dictyostelium*. Unicellular yeasts also possess gsk3-like kinases and Arm repeat proteins but the relationship between these molecules is unknown.

1.7 The system: *Dictyostelium discoideum*

1.7.1 Aggregation of amoebae in response to starvation

The cellular slime mould *Dictyostelium discoideum* is a simple multicellular eukaryote in which to study the process of pattern formation. Single-celled amoebae, when starved, aggregate together in response to extracellular cyclic AMP (cAMP). A few cells form signalling centres and begin to secrete nanomolar pulses of cAMP, which are detected by the surrounding cells. These cells respond in turn by synthesising and secreting their own cAMP, a process known as cAMP relay (reviewed in (Reymond *et al.*, 1995)). Reception of the cAMP signal is via the cell surface G-protein coupled cAMP receptor cAR1, one of a family of four serpentine cAMP receptors present in *Dictyostelium* (Johnson *et al.*, 1993; Louis *et al.*, 1994; Saxe *et al.*, 1993; Saxe *et al.*, 1991). Activation of cAR1 leads to G-protein mediated upregulation of an adenylyl cyclase that synthesises cAMP for secretion. The receptor is rapidly desensitised to the cAMP signal, which leads to downregulation of adenylyl cyclase activity. Furthermore, extracellular cAMP is continuously degraded by a phosphodiesterase. cAMP relay results in a field of cells co-ordinately secreting oscillatory cAMP pulses with approximately 6 minute periodicity. Pulsatile cAMP signalling has several effects in addition to propagating the relay (reviewed in (Firtel, 1996; Reymond *et al.*, 1995)). cGMP levels in the cell increase rapidly, and lead to periodic cell movement by chemotaxis along a gradient of extracellular cAMP, towards the signalling centre. The cells adhere to one another in directional streams and coalesce into aggregates of approximately 10^5 cells.

Pulses of cAMP also lead to changes in transcription, inhibiting growth specific gene expression and activating aggregation-specific genes, such as components of the relay and aggregation-specific adhesion molecules ((Firtel, 1996) and section 1.7.5). Within the aggregate, the extracellular cAMP level is assumed to be high (micromolar) and contributes to repression of the early aggregation genes and to induction of genes specific to multicellularity. "Multicellular" genes induced by cAMP include the three remaining cAMP receptors (Johnson *et al.*, 1993; Louis *et al.*, 1994; Saxe *et al.*, 1993), and two co-ordinately regulated cysteine proteinases (Pears and Williams, 1985; Pears and Williams, 1987; Pears and Williams, 1988). Transcription of these cysteine proteinase genes is upregulated by GBF, a zinc finger transcription factor that binds to short G/C rich sequences within their promoters (Hjorth *et al.*, 1990; Pears and Williams, 1987; Pears and Williams, 1988; Schnitzler *et al.*, 1994). GBF co-ordinately

upregulates expression of a variety of other prestalk- and prespore-enriched genes, and *gbf* cells cannot proceed beyond the early aggregate stage of development (Schnitzler *et al.*, 1994).

1.7.2 Induction of prestalk and prespore cells

A wild type *Dictyostelium* fruiting body consists of 20% stalk cells and 80% spores. The induction of prespore and prestalk cells in the mound, followed by their regulated sorting out into a defined pattern, is a surprisingly complex process, which involves co-ordinated regulation of gene expression and cell adhesion. In addition to cAMP, another secreted factor, differentiation inducing factor (DIF) plays a key role in the process of stalk cell differentiation (Berks and Kay, 1990; Kay and Jermyn, 1983). DIF synthesis can be stimulated by millimolar extracellular cAMP and DIF is present in aggregates and slugs but not growing cells (Brookman *et al.*, 1982). DIF induces the transcription of prestalk-specific genes (Berks and Kay, 1990; Jermyn *et al.*, 1987; Williams *et al.*, 1987) and inhibits prespore-specific gene expression (Berks and Kay, 1990; Early and Williams, 1988). Amoebae plated in low density monolayer culture can be induced to form mature stalk cells in the absence of cell-cell contacts simply by incubation with millimolar levels of cAMP and micromolar DIF, providing a system in which to study cAMP and DIF-mediated signal transduction (Harwood *et al.*, 1995; Jermyn *et al.*, 1987). Initial incubation of amoebae with cAMP renders them competent to respond to DIF, which induces prestalk and stalk cell differentiation. The response to DIF can be inhibited by the continued presence of cAMP (Berks and Kay, 1988; Berks and Kay, 1990; Harwood *et al.*, 1995). However, this system does not provide any information about how the patterning of stalk and spore cells occurs within the developing structure.

Prespore cells, and most prestalk cells, show a scattered distribution in early aggregates. A population of prestalk cells then moves to the top of the aggregate, to form a tip, due to their increased chemotactic motility to cAMP (figure 1.3; (Early *et al.*, 1995; Williams *et al.*, 1989)). The tip secretes cAMP throughout development, and tips from slugs and fruiting bodies can direct the movement of amoebae or disaggregated slug cells (Rubin, 1976; Rubin and Robertson, 1975; Sternfeld and David, 1981). The tip of the aggregate elongates forming a standing slug, which falls over and migrates under conditions of low light, high humidity and high ionic strength. The prestalk cells from the tip of the aggregate form the anterior one fifth of the slug, which stain with vital dyes such as neutral red (Sternfeld and David, 1982),

while most of the posterior four fifths of cells are prespore (figure 1.3). The tip of the slug is proposed to emit a signal which inhibits the formation of secondary tips (Durston, 1976; Sternfeld and David, 1981). At the slug stage differentiation is regulative; and a cell's fate is not irreversible (Abe *et al.*, 1994; Harwood *et al.*, 1991; Rubin and Robertson, 1975; Sternfeld and David, 1981; Sternfeld and David, 1982). Around 10% of scattered cells in the posterior are prestalk cells - the anterior-like cells or ALC's (figure 1.3; (Sternfeld and David, 1981; Sternfeld and David, 1982)). If the tip of a slug is removed, the ALC's move forward to form a new tip (Sternfeld and David, 1981).

The prestalk cells of the slug are heterogeneous, as shown from the study of the regulation of two prestalk-specific genes, *ecmA* and *ecmB*. By transforming *Dictyostelium* with promoter sequences from these genes coupled to reporter genes a previously unexpected variety of distinct prestalk cell populations was discovered (Jermyn *et al.*, 1989; Williams *et al.*, 1989). *ecmA* is expressed by scattered cells of early aggregates, and is highly enriched in the anterior 10% of slug cells, defined as the prestalk A cells (figure 1.3; (Early *et al.*, 1995; Early *et al.*, 1993; Jermyn *et al.*, 1989; Jermyn and Williams, 1991; Williams *et al.*, 1987)). EcmA is a repeat-containing extracellular matrix protein which is secreted into the slime sheath of the slug and the stalk tube of the culminant (McRobbie *et al.*, 1988). The expression profile of *ecmA* mRNA during development is very similar to the accumulation of DIF, and *ecmA* transcription is stimulated by DIF *in vitro* (Brookman *et al.*, 1982; Jermyn *et al.*, 1987; Williams *et al.*, 1987). The rear half of the prestalk zone of the slug contains cells that express *ecmA* at a much lower level than the extreme tip, using a distinct part of the *ecmA* promoter (figure 1.3; (Early *et al.*, 1995; Early *et al.*, 1993; Jermyn *et al.*, 1989; Jermyn and Williams, 1991)). These cells have been named prestalk O cells. A subset of ALC's share the properties of these prestalk O cells, as they also express low levels of *ecmA* (Early *et al.*, 1993). Prestalk A cell formation in monolayers requires ten-fold higher levels of DIF than prestalk O cells (Early *et al.*, 1995). In addition, a further subset of prestalk cells express a protein highly related to ecmA, called ecmB (Gaskell *et al.*, 1992; Jermyn *et al.*, 1989). *ecmB* induction requires higher levels of DIF than *ecmA* (Berks and Kay, 1990; Jermyn *et al.*, 1987). The *ecmB*-expressing cells, termed prestalk B cells, arise in a scattered fashion in early aggregates but become concentrated to the base of the mound as the tip forms (figure 1.3; (Williams *et al.*, 1989)). In the slug, a proportion of prestalk B cells in the posterior, the ALC's, also express *ecmA* (Gaskell *et al.*, 1992). A second population of *ecmB*-expressing cells, known as

prestalk AB cells, arises later in development, using a distinct region of the *ecmB* promoter from that expressed in ALC's (figure 1.3). These cells form a cone-shaped stalk tube primordium within the prestalk A/O region of the slug, and initiate stalk tube formation when a fruiting body is formed (Ceccarelli *et al.*, 1991; Gaskell *et al.*, 1992; Jermyn *et al.*, 1989; Jermyn and Williams, 1991; Sternfeld, 1992).

1.7.3 A gsk3ß homologue is required for correct cell type proportioning in the aggregate

As in metazoa, the *Dictyostelium* homologue of gsk-3ß, *gskA*, is critical for regulation of cell fate (Harwood *et al.*, 1995). A proportioning decision is regulated by gskA in the aggregate. gskA is not required to induce the formation of prestalk or prespore cells. However, the *gskA*⁻ mutant overproduces prestalk B cells at the expense of the prespore cells, resulting in a fruiting body with an expanded basal disc supporting a malformed stalk and very few spores (Harwood *et al.*, 1995). A similar phenotype was seen when cells were developed in the presence of lithium ions, which inhibit gskA/gsk3ß (Maeda, 1970). Thus gskA acts to negatively regulate the prestalk B cell pathway. The DIF induction of stalk cells in *gskA*⁻ mutant monolayers cannot be inhibited by the presence of extracellular cAMP (Harwood *et al.*, 1995). Importantly, cAMP activates gskA at the mound stage, and this activation of gskA is mediated by cAR3 (Plyte *et al.*, 1999). The *cAR3*⁻ mutant has a similar phenotype to the *gskA* mutant, overexpressing *ecmB* in the mound and producing cAMP-insensitive stalk cells in a monolayer (Plyte *et al.*, 1999). However, the *cAR3*⁻ mutant morphology is not as severe as that of *gskA*⁻, suggesting that another cAMP receptor, such as cAR1, may be able to substitute in part for cAR3 (Johnson *et al.*, 1993; Plyte *et al.*, 1999). Evidence exists that cAR4, a prestalk-enriched receptor with lower affinity for cAMP than cAR3 (Louis *et al.*, 1994), also regulates gskA activity (Ginsburg and Kimmel, 1997). *cAR4*⁻ mutants have a reduction in prestalk B differentiation and increased prespore differentiation (Ginsburg and Kimmel, 1997; Louis *et al.*, 1994). Activation of cAR4 is thought to stimulate prestalk cell differentiation via its inhibition of gskA activity (Ginsburg and Kimmel, 1997). Development of *cAR4*⁻ cells in the presence of lithium ions rescues their prestalk/prespore patterning defect back to near wild type levels (Ginsburg and Kimmel, 1997). However, *cAR4*⁻ cells also show reduced prestalk A cell induction *in vivo* and in response to DIF, suggesting cAR4 has gskA-independent functions. cAR2 may function redundantly with cAR4 at the mound stage as *cAR2*⁻ mutants also show increased prespore and reduced *ecmB* gene expression (Saxe *et al.*,

1993). cAR2 must also have other developmental functions as *cAR2*⁻ mutants arrest as mounds (Saxe *et al.*, 1993), whereas *cAR4*⁻ mutants undergo culmination (Louis *et al.*, 1994).

The proposed regulation of gskA by cAR3 and cAR4 could be analogous to the inhibition, in most metazoa, and activation, in *C. elegans*, of gsk3ß activity by Wnt signals, acting on serpentine Fz receptors, during metazoan development (section 1.2). In addition, regulated ubiquitination and degradation of proteins may be required during later developmental stages, as a mutation in a ubiquitin-conjugating enzyme delays development after the mound stage and abolishes culmination (Clark *et al.*, 1997). The similarity of these processes with Wnt signalling raises the question of whether a *Dictyostelium* ß-catenin/Arm homologue functions downstream of gskA, and is regulated by ubiquitination and degradation, as in metazoa.

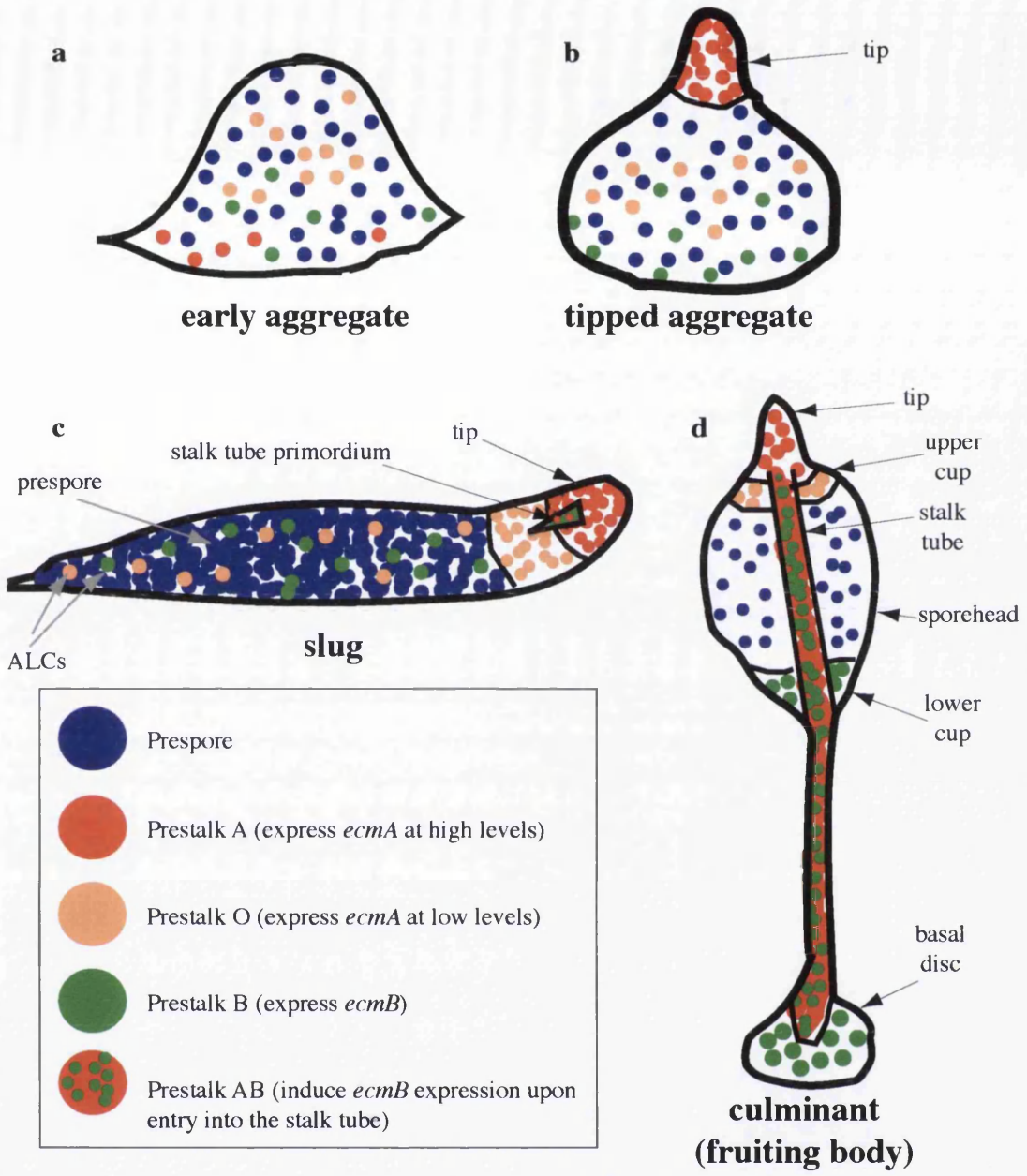

Figure 1.3 Cell differentiation and movement during *Dictyostelium* development
a) Early aggregates consist of 80% scattered prespore cells and 20% prestalk cells.
b) The prestalk A cells, defined by high levels of *ecmA* mRNA expression, move to the top of the aggregate to form a tip.
c) The anterior one fifth (tip) of the slug consists of prestalk A cells and prestalk O cells. Prestalk O cells express lower levels of *ecmA* than the prestalk A cells in the extreme tip. In addition a central cone is present within the tip and this contains cells expressing *ecmA* and *ecmB* (prestalk AB cells). These cells initiate stalk tube formation at culmination. The posterior four fifths of the slug contain prespore cells and a population of anterior-like cells (ALCs). ALCs are prestalk cells that express *ecmA*, similarly to the prestalk O cells, and/or *ecmB*.
d) At culmination, prestalk A cells, and later some prestalk O cells, move from the tip down into the stalk tube and induce stalk-specific *ecmB* expression. ALC's move to surround the developing spores, forming the upper and lower cups. The remaining *ecmB*-expressing ALC's form the basal disc, which anchors the culminant to the substratum.

1.7.4 Cell movements and terminal differentiation during culmination

How do cells in the slug move to form a mature fruiting body with an axis running from the tip to the base of the central stalk tube? During slug migration the stalk tube primordium is periodically jettisoned from the rear of the slug and reformed in the tip (Sternfeld, 1992). At culmination, which is induced by a reversal of the conditions favouring slug migration, the slug stands on end and prestalk cells in the tip migrate down through the developing prespore mass whilst secreting cellulose. The prestalk AB cells, which form the stalk tube primordium, migrate first, followed by the prestalk A cells and a subset of prestalk O cells (Early *et al.*, 1993; Jermyn and Williams, 1991; Sternfeld, 1992). In this manner the cellulose-encased stalk elongates, lifting the spore mass away from the substratum. Two subsets of ALC's move in opposite directions to form the basal disc, which anchors the fruiting body to the substratum, and the upper and lower cups which cradle the top and bottom of the sporehead (figure 1.3; (Dormann *et al.*, 1996; Jermyn *et al.*, 1996; Jermyn and Williams, 1991; Sternfeld and David, 1982)). Some prestalk O cells also become part of the upper cup (Early *et al.*, 1993). Once cells enter the stalk tube, they terminally differentiate to form vacuolated stalk cells and the surrounding prespore cells form mature spores. How do stalk and spore cells terminally differentiate within the culminant? Cells isolated from the anterior 10% of a slug can form stalk cells without the addition of exogenous factors, and tips can induce other dissociated slug cells to become stalk (Town and Stanford, 1977). Although DIF induces stalk cell formation in monolayers, DIF alone is not responsible for triggering terminal differentiation of stalk tube stalk cells *in vivo*.

Two direct TTGA repeats are required for DIF activation of a minimal *ecmA* promoter construct (Kawata *et al.*, 1996). When prestalk A cells enter the stalk tube, they begin to express *ecmB* (figure 1.3), using a stalk tube (ST)-specific region of the *ecmB* promoter (Ceccarelli *et al.*, 1991). Transcription of the *ecmB* gene from this region of the promoter is normally repressed in all prestalk cells, apart from the prestalk AB cells in the stalk tube primordium, prior to culmination (Ceccarelli *et al.*, 1991; Harwood *et al.*, 1993). Interestingly, the repressor elements are two inverted repeats identical in sequence to the direct repeats of the *ecmA* activator (Kawata *et al.*, 1996). When the repressor elements are introduced into the *ecmA* promoter, they prevent its expression in the prestalk cells outside the stalk tube (Harwood *et al.*, 1993). In addition, weakening of these elements results in expression of *ecmB* outside the stalk

tube during culmination, but continued repression of *ecmB* in the slug (Harwood *et al.*, 1993). This suggests that a signal that induces derepression of *ecmB* in the stalk tube may increase in strength at culmination. Repression of *ecmB* can be lifted by the activity of the cAMP-dependent protein kinase (PKA). Although PKA has functions earlier in development (Harwood *et al.*, 1992a; Hopper and Williams, 1994; Mann *et al.*, 1997), its specific inhibition in prestalk cells, using a dominant negative form which cannot bind cAMP, blocks *ecmB* expression, culmination and stalk cell terminal differentiation (Harwood *et al.*, 1993; Harwood *et al.*, 1992b). *ecmB* reporter constructs lacking the repressor element are expressed in the absence of PKA activity, showing that PKA activity specifically controls the activity of the repressor element (Harwood *et al.*, 1993). A *Dictyostelium* STAT protein (DdSTATa) has been identified which binds to both the repressor and activator repeats *in vitro* (Kawata *et al.*, 1997). Tyrosine phosphorylation and nuclear localisation of DdSTATa is induced by extracellular cAMP and cAR1 during late aggregation; in the slug DdSTATa is localised to the nuclei of tip cells (Araki *et al.*, 1998). Indeed, DdSTATa appears to be the *in vivo* repressor of *ecmB*, as *DdSTATa* mutants express stalk tube *ecmB* throughout the tip of the slug (Mohanty *et al.*, 1999).

How is PKA activity regulated in prestalk cells during culmination? A signal must exist which elevates the level of intracellular cAMP and activates PKA as prestalk cells enter the stalk tube (Harwood *et al.*, 1992b; Hopper *et al.*, 1993a; Yamada and Okamoto, 1994). The nature of this signal is at present unknown. However a low molecular weight factor, STIF, has been characterised. This synergises with DIF to induce stalk cell formation and stalk tube-specific *ecmB* expression in isolated cells (Yamada *et al.*, 1997). Furthermore, inhibition of PKA blocks the activity of STIF (Yamada *et al.*, 1997). Mutations in an intracellular histidine kinase, rdeA, or a phosphodiesterase, regA, cause accelerated terminal differentiation due to permanently elevated levels of intracellular cAMP (Chang *et al.*, 1998; Thomason *et al.*, 1998). When activated, rdeA phosphorylates, and is likely to activate, regA (Thomason *et al.*, 1999). Thus activation of rdeA would inhibit culmination and terminal differentiation. Ammonia has been demonstrated to antagonise the effects of DIF and inhibit culmination; this may be due to its elevating the intracellular pH of prestalk cells, as stalk cell maturation can be induced by lowering the intracellular pH (Gross *et al.*, 1983; Inouye, 1988; Schindler and Sussmann, 1977). Ammonia could also affect PKA activity by inhibiting the accumulation of intracellular cAMP (Schindler and Sussman, 1979).

Additional PKA-independent signals may also be required to induce complete terminal differentiation of stalk cells. The inhibition of stalk cell formation by extracellular cAMP (Berks and Kay, 1988; Berks and Kay, 1990; Harwood *et al.*, 1995) is independent of PKA (Harwood *et al.*, 1995; Hopper *et al.*, 1993a). Therefore a drop in extracellular cAMP concentration, as prestalk AB cells move away from the tip into the stalk tube, could contribute to their terminal differentiation. The cellulose stalk tube may contribute to the terminal differentiation of stalk cells, as enclosure in artificial cellulose tubes can induce preculminant cells to become stalk cells (Farnsworth, 1974). Cellulose itself could signal to prestalk cells, or the existence of the stalk tube could lead to a localised concentration of a differentiation-inducing signal, such as the peptide factor SDF-1, which is released during culmination and can stimulate stalk cell formation (Anjard *et al.*, 1998a). However, anterior-like cells that form the basal disc terminally differentiate without inclusion in a stalk tube. These data suggest that regulation of terminal stalk cell differentiation may vary in different prestalk cell populations. Indeed, a gene expressed in terminally differentiated stalk cells, *staB*, has been characterised, which requires discrete promoter elements for its expression in stalk cells deriving from different prestalk populations (Robinson and Williams, 1997). The phenotype of the *cudA* mutant, which cannot culminate, also suggests that the complete terminal differentiation of stalk cells is a multistep process. *cudA* prestalk cells express stalk-specific *ecmB* and *staB* and secrete some cellulose, however they do not vacuolate or form a stalk tube (Fukuzawa *et al.*, 1997).

PKA is also required for terminal spore differentiation. Cells containing constitutively active PKA show accelerated terminal spore, as well as stalk, differentiation (Hopper *et al.*, 1993a; Simon *et al.*, 1992) and inhibition of PKA in prespore cells blocks their terminal differentiation (Hopper *et al.*, 1993b). Cells transformed with a prespore-specific inhibitor of PKA are hypersensitive to inhibition of culmination by ammonia, showing that a rise in intracellular cAMP is also required to activate PKA activity in the prespore to spore transition (Hopper *et al.*, 1993b). Cells mutant for a GATA-like transcription factor, *stkA*, form stalk cells, but no spores, during culmination (Chang *et al.*, 1996). Thus *stkA* could be a downstream effector of PKA signalling in spore cells. Importantly, stalk cell differentiation appears to be a prerequisite for spore cell differentiation. When PKA is inhibited in prestalk cells, cells which attempt to culminate form neither stalk or spore cells (Harwood *et al.*, 1992b). This strongly suggests that an uncharacterised signal from mature stalk cells in the stalk tube is required to induce spore differentiation. The peptide factors SDF-1 and SDF-2

are good candidates for such a signal (Anjard *et al.*, 1998a; Anjard *et al.*, 1997; Anjard *et al.*, 1998b), and a prestalk-specific *myb* transcription factor may be required for their production (Guo *et al.*, 1999).

1.7.5 Cell adhesion systems during pattern formation

How do developing *Dictyostelium* cells adhere to one another? Four developmentally regulated adhesion systems have been defined by their differential sensitivity to disruption by EDTA and EGTA (reviewed in (Bozzaro and Ponte, 1995; Siu *et al.*, 1997)). Generation of monovalent antibodies, which could block the reaggregation of cells dissociated at different stages of development, indicated that each adhesion system was mediated by one or more specific cell surface proteins (Beug *et al.*, 1970; Beug *et al.*, 1973). The earliest system, contact sites B (Beug *et al.*, 1973; Garrod, 1972), which is sensitive to both EDTA and EGTA, is involved in forming the initial contacts between cells during streaming. A few hours later, an EDTA sensitive/EGTA resistant adhesion system is also apparent (Fontana, 1993). Antibodies raised to the contact sites B glycoprotein gp126 inhibit early EDTA/EGTA-sensitive intercellular adhesion and phagocytosis (Chadwick *et al.*, 1984; Chadwick and Garrod, 1983). Another glycoprotein, gp24, has also been implicated in EDTA-sensitive adhesion. gp24 is identical to DdCAD-1, a calcium-binding protein with homology to the extracellular domain of mammalian E-cadherin (Wong *et al.*, 1996). DdCAD-1 is expressed during early development and its expression is upregulated by nanomolar cAMP pulses (Siu *et al.*, 1997). A DdCAD-1 fusion protein blocks EDTA-sensitive adhesion and neutralises the anti-adhesive effect of DdCAD-1 antibodies. Conversely DdCAD-1 antibodies block binding of DdCAD-1 protein to the cell surface, suggesting a homophilic interaction of DdCAD-1 molecules on adjoining cells (Siu *et al.*, 1997; Wong *et al.*, 1996). Unlike classical cadherins, DdCAD-1 is a soluble protein with no transmembrane domain. During early aggregation, DdCAD-1 localises to F-actin-containing cell protrusions, and to contacts between streaming cells (Siu *et al.*, 1997; Wong *et al.*, 1996). Despite its soluble nature, a significant amount of DdCAD-1 is found on the extracellular surface, suggesting the existence of a transmembrane linker protein (Wong *et al.*, 1996). A mammalian cadherin, LI-cadherin, with no catenin-binding cytoplasmic tail is known. This can mediate calcium-dependent cell-cell adhesion but cannot reorganise the actin cytoskeleton (Kreft *et al.*, 1997); perhaps the transient contacts mediated by DdCAD-1 are analogous to those of LI-cadherin.

Once cells have entered the aggregate they no longer express membrane-associated DdCAD-1. Instead an aggregation-specific, EDTA-resistant cell adhesion system, contact sites A, is apparent (Beug *et al.*, 1973). Contact sites A is mediated by gp80, encoded by the *csA* gene. Antibodies to gp80 block EDTA-resistant cell adhesion and cell surface binding of gp80, and *csA*$^-$ cells are excluded from aggregates formed by a mixture of wild type and *csA*$^-$ cells, again suggesting a homophilic mode of adhesion (Bozzaro and Ponte, 1995; Geltosky *et al.*, 1979; Siu *et al.*, 1997). csA is a globular glycoprotein similar to mammalian immunoglobulin superfamily cell adhesion molecules such as N-CAM, which localises to cell-cell contacts within the aggregate (Kamboj *et al.*, 1989; Siu *et al.*, 1997). csA transcription and cell surface expression is stimulated by cAMP pulses (Faix *et al.*, 1992; Gerisch *et al.*, 1975) and inhibited by the disruption of early gp24/DdCAD-1-mediated adhesion (Desbarats *et al.*, 1994). Anti-csA antibodies block streaming and aggregation (Kamboj *et al.*, 1989), although *csA*$^-$ cells can complete culmination on agar (Harloff *et al.*, 1989). However, when developed on soil, to mimic the native habitat of *Dictyostelium*, *csA*$^-$ cells show reduced intracellular adhesion, reduced motility and form fewer fruiting bodies (Ponte *et al.*, 1998). The presence of *csA* confers a selective advantage on wild type cells under these conditions, as mutant cells mixed with wild type cells sort out of the mixes and few *csA*$^-$ spores are recovered from the resulting culminants (Ponte *et al.*, 1998). Overexpression of csA from its own promoter confers very strong EDTA-resistant adhesion on developing cells (Faix *et al.*, 1992). This results in fragmented streams; many cells never enter the culminant but remain on the substratum. Thus the strength of intercellular adhesion must be tightly controlled during development: too little or too much adhesion can lead to a defect in fruiting body formation.

A second, postaggregative, EDTA-resistant adhesion system was uncovered in cells where csA-mediated adhesion was disrupted. Dissociated *csA*$^-$ aggregates can re-adhere in the presence of EDTA, but ~4 hours later than wild type cells (Harloff *et al.*, 1989). Antibodies to gp150 block cell adhesion at the slug stage and gp150 is maximally expressed after the aggregation stage (Geltosky *et al.*, 1979). gp150 may mediate differential adhesion between prestalk and prespore cells. A F_{ab} fragment raised to gp150 blocked the sorting of prestalk cells to the tip of the mound, and inhibited the reassociation of disaggregated prespore cells to a greater extent than prestalk cells (Lam *et al.*, 1981). gp150 may be identical or related to the LagC gene product, a novel prestalk-enriched protein which is expressed from the loose aggregate stage (Dynes *et al.*, 1994; Siu *et al.*, 1997). *lagC*$^-$ mutants can undergo initial aggregation but further

development is disrupted. Cell-type specific gene expression is barely induced and cannot be rescued by extracellular cAMP. Mutant cells disaggregate and then reaggregate to form very small mounds and fruiting bodies, with very few mature stalk or spore cells (Dynes *et al.*, 1994). When mixed with wild type cells, *lagC*$^{-}$ cells can form stalk cells but not spores, suggesting autonomous and nonautonomous roles in development (Dynes *et al.*, 1994).

Virtually nothing is known about intercellular adhesion systems during culmination. Prestalk and prespore cells from dissociated slugs show differential cohesiveness and resistance to EDTA (reviewed in (Bozzaro and Ponte, 1995; Siu *et al.*, 1997), but the molecules involved in this process are uncharacterised. Culmination involves cell movements, structural rearrangements and adhesion between many different cell types, but the blocking antibody techniques used to discover aggregation-specific cell adhesion molecules have not been used successfully at the later, more complex stages of development. When this work was started, no intercellular junctional structures analogous to those found in developing animal tissues had been discovered (Johnson *et al.*, 1977). Although *Dictyostelium* contains proteins with some similarity to mammalian adhesion molecules, such as csA, DdCAD-1 and a talin homologue, which may mediate adhesive changes during tip formation (Kamboj *et al.*, 1989; Tsujioka *et al.*, 1999; Wong *et al.*, 1996), no transmembrane cadherin-like molecule which could bind to catenins is presently known. Genetic loss of function studies are likely to be the only way to discover the molecules involved in adhesive interactions in the culminant.

1.7.6 Can cell-cell contact regulate gene expression in *Dictyostelium*?

Cells can be induced to differentiate into stalk and spore in the absence of cell-cell contact (section 1.7.2), but does cell-cell contact play more than just a passive adhesive role within the developing organism? Early cell-cell contacts may help to reinforce the cAMP secretion/relay response at the start of aggregation: physical contact between *Dictyostelium* amoebae, or between amoebae and bacteria, elicits cAMP secretion and the authors propose that this may be brought about by cytoskeletal deformation (Fontana *et al.*, 1991). Interestingly, contact with a small number of amoebae diminishes the cAMP relay response, while contact with bacteria, high density amoebae or the membranes of aggregation-competent cells increases the secretion of cAMP in response to the initial cAMP signal (Fontana *et al.*, 1991).

Some evidence exists that cell-cell contact can mediate gene expression. Overexpression of csA in vegetative cells causes them to aggregate and express developmental genes (Faix *et al.*, 1990) although gene activation could be an indirect effect due to starvation of cells within such aggregates. Disaggregation of slug cells causes rapid loss of mRNAs for prestalk- and prespore-specific genes (Mehdy *et al.*, 1983) and for a developmentally regulated enzyme involved in cellulose biosynthesis (Haribabu *et al.*, 1986). This certainly suggests that multicellularity may be required to maintain gene expression during development. However, mRNA expression can be partially rescued by the addition of micromolar cAMP to disaggregated cells, suggesting the need for cell contact can be bypassed, as in monolayer cultures (Chisholm *et al.*, 1984; Haribabu *et al.*, 1986; Mehdy *et al.*, 1983). It has also been proposed that cell-cell contact, in addition to pulsatile cAMP, is necessary for prespore gene induction. When cells are starved in high speed shaken suspension or in the presence of EDTA (to inhibit cell contacts), prespore gene expression cannot be induced by micromolar cAMP alone (Chisholm *et al.*, 1984; Mehdy *et al.*, 1983). Most of these experiments were performed using rapidly shaken cells, where it is assumed that shear forces prevent cell-cell contacts from occurring. However the shaking speeds, cell densities and degree of residual cell aggregation varies between experiments. A more comprehensive study showed that *csA* expression was severely reduced when gp24/DdCAD-1-mediated adhesion was blocked by EDTA, carnitine or fast-shaken low density culture (Desbarats *et al.*, 1994). Application of exogenous cAMP pulses to cells treated in these ways could not restore wild type levels of *csA* expression (Desbarats *et al.*, 1994). It is interesting that the early DdCAD-1 adhesion system appears to be required for full induction of csA-mediated adhesion to occur.

1.8 Revisiting old problems

I aim to elucidate whether cell-cell adhesion and gene expression are co-regulated by the presence of a ß-catenin-like molecule in *Dictyostelium.* In doing so, I hope to provide data which extend or complement what is known about the link between developmental signalling and cell-cell adhesion in metazoa.

Therefore, the aims of this work are to identify and isolate ß-catenin homologues in *Dictyostelium*, to analyse whether they are developmentally regulated, to investigate whether they are associated with the actin cytoskeleton, and to investigate whether they are regulated by gskA.

Chapter 2

Materials and methods

2.1 *Dictyostelium* culture and transformation

2.1.1 Cell culture and development

Vegetative cells were grown at 22°C in association with *Klebsiella aerogenes* on SM, or in shaking culture in axenic medium with 0.1mg/ml streptomycin and appropriate antibiotic selection. For development, cells were harvested from bacterial clearing plates (10^6 cells seeded on 10cm diameter SM/*Klebsiella* plates and grown for 48 hours) or spun down from logarithmically growing culture (1-2x10^6 cells/ml) and washed four times in KK_2. Cells were developed by incubation at 22°C in a humid atmosphere for the required length of time: 5x10^7 cells were plated onto 10cm diameter 1.8% KK_2 agar plates, or 1x10^7 cells were plated on 4.7cm diameter, 0.45µm pore size, nitrocellulose filters (Millipore) on a KK_2-soaked prefilter.

For long term storage, 10^7 cells or more were resuspended in 1ml horse serum containing 5% DMSO in a 2ml cryovial (Nunc) and frozen down slowly to –80°C in a thick-walled airtight polystyrene container before transferring to liquid nitrogen stores.

2.1.2 DNA transformation of *Dictyostelium* cells

2.1.2(i) Electroporation

This method was used for generating blasticidin-resistant gene knockout strains and for overexpressing Aar in *Dictyostelium*. Logarithmically (1-2x 10^6/ml) growing cells from axenic culture were harvested and washed in sterile, chilled electroporation buffer (KK_2, 50mM sucrose). 10^7 Cells were resuspended in 800µl electroporation buffer per 0.4cm cuvette (Flowgen). 20µg of linearised plasmid DNA (for knockouts) or 10µg uncut plasmid DNA (for overexpression) was added and the cuvette chilled on ice for 10 min. Electroporation was carried out at 1.6kV, 3µF (BioRad electroporator) with time constants of around 0.4ms. The cells were allowed to recover on ice for a further 10 min before adding 1mM $MgCl_2$/1mM $CaCl_2$ and incubating at room temperature for 15 min. The contents of each cuvette was plated out on to four 10cm plates containing 10ml axenic medium and left overnight. The next day selection was commenced in axenic medium supplemented with heat-killed bacteria (*E.coli* b/r-see section 2.1.2(ii)) and the appropriate selection (10µg/ml blasticidin S (ICN) or 20µg/ml G418 (Gibco)). After a further 2-3 days selection was continued in axenic medium and without b/r. Medium was changed every 1-3 days. Plates of homologous recombinants were grown to confluence and cloned out onto SM/*Klebsiella* at a density of 50-100

cells per plate. Plasmid transformant colonies were cloned out into 24 well plates (Falcon).

2.1.2(ii) Calcium phosphate-mediated transformation

This method was used to generate high copy number random integrations of plasmid DNA into the *Dictyostelium* genome. $2x10^7$ cells were used per transformation. 10ml of axenically growing cells (10^6/ml) were plated onto 10cm tissue culture dishes and allowed to settle for 10 min. The axenic medium was then replaced with HEPES-HL5 medium and cells were left to equilibrate for a minimum of 2 hours. The DNA precipitate (for 2 plates of cells) was made by mixing 600µl 2x HEPES-buffered saline (HBS), 24µg plasmid DNA and ddH_2O to 1.2ml. Whilst vortexing this mixture gently, 76µl of 2M $CaCl_2$ was added dropwise. The mixture was then vortexed hard and left for 30 min at room temperature. 600µl of DNA precipitate was added dropwise to a plate of cells and incubated for at least 4 hours. Cells were then osmotically shocked with 2ml of 15% glycerol in HBS for 2 min. The glycerol/HBS was then replaced with non-selective axenic medium supplemented with heat killed *E.coli* b/r overnight. The next day selection was commenced: 20µg/ml G418 in axenic medium/*E.coli* for three days then selective medium without bacteria thereafter. Transformant colonies were grown to confluence on plates and used as pools for ß-galactosidase staining experiments.

Sterile heat-killed bacteria were made as follows: a 1l overnight culture of *E.coli* b/r was pelleted (5000g, 5 min) and washed three times in 1l KK_2. Cells were resuspended in KK_2/2mM $MgSO_4$ to a final concentration of $9x10^{10}$ cells/ml (an OD_{650} of $0.1=10^8$ cells/ml). Cells were heat-killed by placing at 75°C for 20 min and stored in aliquots at –20°C for use 1:100 in cell culture.

2.2 Immunological procedures

2.2.1 SDS PAGE and western blotting of whole cell extracts

Cells were scraped from KK_2 agar plates at different stages of development, washed in KK_2 and pelleted at 2000g for 2 min. For SDS-PAGE, cells were lysed by boiling for 5 min in Laemmli buffer and debris pelleted at 20 000g for 5 min before loading on an acrylamide gel. 10^6 cell equivalents per lane were loaded on 7.5% resolving/4.5% stacking acrylamide gels (37.5:1 acrylamide:bisacrylamide; Protogel, National Diagnostics Corporation), and run at 150V for 1.5 hours using standard procedures (Sambrook *et al.*, 1989) with broad range molecular weight standards (BioRad). Bacteria were lysed in the same way and extracts run out on 12% resolving

gels. Some gels were then stained with Coomassie Blue overnight and destained over several hours with 4-5 changes of destain to visualise total protein. Other gels were equilibrated in transfer buffer (48mM Tris, 39mM glycine, 20% methanol) and protein was electrophoretically transferred to Hybond C-extra nitrocellulose membrane (Amersham Pharmacia Biotech) by semi-dry blotting in a BioRad transfer cell according to the manufacturer's instructions. The membrane was rinsed in phosphate buffered saline (PBS) and proteins were visualised using 2% PonceauS in 1% acetic acid to ensure even transfer and loading. Membranes were blocked for 1 hour at room temperature or overnight at 4°C in 10% dried skimmed milk, 0.1% Tween-20. After five washes in PBS/0.1% Tween-20 (PBST) membranes were incubated in primary antibody diluted in PBST for 1-2 hours at room temperature (for anti-Aar antiserum, overnight, 4°C, 5% milk/PBST), washed four times and incubated in secondary antibody diluted in PBST for a further hour. After further washing the membrane was sealed in Saran wrap (Dow Chemical); the secondary antibody was detected using enhanced chemiluminescence (ECL, Pierce) according to the manufacturer's instructions and exposure to Biomax (Kodak) film. For stripping and reprobing, membranes were incubated at room temperature for 10 min in 0.1M glycine pH 2.5 followed by neutralisation in 1M Tris pH 8.0.

2.2.2 Cell fractionation.

Dictyostelium cells were sonicated into homogenisation buffer (20mM Tris pH 7.5, 250mM sucrose, 100mM EGTA, 20mM EDTA), containing a cocktail of protease inhibitors (1μM AEBSF, 10mg/ml CLAP, 10mM benzamidine, 10mM DTT) before spinning into cytosol (S100) and pellet (P100) fractions at 100 000g for 30 min at 4°C. The pellet was further fractionated in homogenisation buffer containing 1% TritonX-100 or 1% Nonidet-P40 to give detergent-soluble (Ps) and insoluble (Pi) fractions (10 000g, 30 min, 4°C). Fractions were analysed by SDS-PAGE and western blotting as before, loading 10^5-10^6 cell equivalents per lane.

2.2.3 Antisera

The following antibodies and antisera were used during the course of this work:

- Anti-HPGN (rabbit antiserum from Drs. H. Cordingley and A. Magee: MRC NIMR, London, UK), raised against a fusion protein consisting of the N-terminus of human plakoglobin (amino acids 5-304) C-terminal to glutathione-S-transferase (GST) (Cordingley, 1996). This was used at 1:5000 in PBST for western blotting.

- Anti-XPGN (Cordingley, 1996), as HPGN, against a GST-fusion of *Xenopus* plakoglobin (amino acids 1-106) and used at 1:5000 in PBST for western blotting.
- Anti-XPGC (Cordingley, 1996), as XPGN but amino acids 666-738 of *Xenopus* plakoglobin, used at 1:2000 in PBST for western blotting.
- Anti-Armadillo (rabbit antiserum from Dr. L. Ruel: University of Toronto, Canada) against the C-terminal 35-40kDa of *Drosophila* Armadillo, used at 1:500 in PBST for western blotting.
- VB2 (affinity purified rabbit antiserum from Dr. V. Braga: MRC-LMCB, London, UK) raised to the C terminal peptide from human ß-catenin (amino acids 768-781, sequence PGDSNQLAWFDTDL) (Braga *et al.*, 1995). This was used at 1:1000 in PBST for western blotting.
- C2206 (Sigma) against the C-terminal peptide (amino acids 768-781) of human/mouse ß-catenin: PGDSNQLAWFDTDL, used at 1:5000 in PBST for western blotting.
- MUD-1, a monoclonal antibody against the psA protein product (Early *et al.*, 1993; Krefft *et al.*, 1983) was used 1:100 in western blots.
- Mouse anti-Xpress (Invitrogen) used 1:5000 in PBST for blotting bacterial cell extracts.
- Donkey anti-rabbit horseradish (hrp) peroxidase conjugate (Amersham Pharmacia Biotech), used 1:16 000 in PBST.
- Goat anti-rabbit-hrp conjugate (Vector Laboratories) used at 1: 20 000 in PBST.
- Horse anti-mouse-hrp conjugate (Vector Laboratories) used 1:10 000 in PBST
- Goat anti-rabbit and mouse-TRITC conjugates preabsorbed against *Dictyostelium* cells and methanol were a gift from Dr. R. Ginger (MRC-LMCB, London, UK)
- Anti-Aardvark preimmune serum was tested at 1:50, 1:100 and 1:500 for immunofluorescence. Preimmune and immune sera were also tested on western blots at 1:100, 1:500, 1:1000 and 1:5000 in PBST and in 2% and 5% milk/PBST.

2.2.4 Immunoabsorption and immunodepletion

VB2 and HPGN were immunoabsorbed against an acetone powder made from vegetative *Dictyostelium* cells before use (Harlow and Lane, 1988). Briefly, 10^7 cells were suspended in 2ml ice-cold KK_2 and 8ml -20°C acetone was added. This suspension was left on ice for 30 min with occasional vigorous mixing. The precipitate was collected by spinning at 10 000g for 10 min, resuspended in more acetone on ice for 10 min, and pelleted again. The resulting precipitate was air-dried to a powder at room temperature between two sheets of Whatman 3MM paper. The powder was added to antisera to a final concentration of 1% and incubated at 4°C for 30 min before pelleting debris (10 000g, 10 min) and using the supernatant as normal antiserum.

HPGN was also immunoabsorbed against glutathione-agarose beads (G4510, Sigma). To test specificity on blots, HPGN was immunodepleted with the protein (on glutathione agarose, section 2.2.5) to which it was raised (Cordingley, 1996). Similarly VB2 was preincubated with the peptide to which it was raised (from Dr. V. Braga, MRC-LMCB, London, UK) for 30 min at room temperature before use.

2.2.5 Preparation of a GST-HPGN fusion protein for immunodepletion

Bacteria containing the GST-HPGN expression plasmid (Cordingley, 1996) were a gift from Drs H. Cordingley and A. Magee (MRC NIMR, London, UK). A 100ml starter culture in L-Broth was grown overnight at 37°C, diluted to 1 litre and grown further at 37°C to an OD_{600} of 0.5-0.6. Fusion protein expression was then induced by the addition of 0.1mM IPTG for 5 hours at 37°C. Bacteria were pelleted at 6000g for 5 min at 4°C and subjected to sonication-lysis on ice in 3ml Buffer A supplemented with 1µM AEBSF, 10mg/ml CLAP, 1mM DTT. Bacterial debris were pelleted at 10 000g for 10 min at 4°C and the supernatant containing the fusion protein was added to 1ml of packed Glutathione-agarose beads (G4510, Sigma) previously equilibrated in several volumes of buffer A. The supernatant and beads were incubated with agitation at 4°C for 30 min, then the slurry was transferred to a 10ml gravity flow column (BioRad) at 4°C. The beads with bound fusion protein were allowed to settle and the flowthrough was discarded. Beads were washed in 20ml of ice-cold Buffer A/1mM DTT and then with 5ml Buffer A alone. To immunodeplete anti-HPGN, 1ml of antiserum diluted 1:10 in PBS was passed over the beads at 4°C and the flowthrough was collected in 250µl fractions. 10µl of each fraction was analysed on a Coomassie-stained SDS-PAGE gel and a portion of one IgG-containing fraction was diluted 1:500 in PBST and used to probe western blots.

2.2.6 Immunofluorescent cell staining

Vegetative cells were allowed to settle on glass coverslips (Sigma) or multiwell slides (ICN). Streaming/aggregating cells were obtained by placing cells on dishes containing coverslips and developing them for 12-14 hours at room temperature under KK_2. To fix the cells, slides and coverslips were incubated in ice-cold methanol for 10 min. Cells were washed 3 times in PBS and blocked against non-specific binding in 1% BSA for at least 30 min at room temperature. Cells were incubated in primary antibody or preimmune serum for 3-4 hours at room temperature in a damp atmosphere. 3 more washes were then performed and secondary antibody was added for 1-2 hours at room temperature before 3 final washes in PBS and one in ddH_2O. Slides and coverslips were air-dried before mounting and viewing on a Zeiss Axioskop fluorescence microscope.

2.3 Blotting of DNA and RNA

2.3.1 Preparation of genomic DNA from *Dictyostelium*

10^9 Dictyostelium cells were harvested from shaking axenic culture or SM *Klebsiella* plates, washed in KK_2 and shaken in KK_2 at $2\text{-}4x10^7$/ml for at least 4 hours. Starved cells were pelleted and resuspended in KK_2 at $2\text{-}4x10^8$/ml. Cells were lysed to release intact nuclei by adding 40ml of ice-cold cell lysis buffer and mixing. Nuclei were pelleted at 4000g for 10 min, washed and resuspended in 0.3ml lysis buffer. Proteinase K (Sigma) was added to 100µg/ml in 10ml digestion and the mixture incubated at 60°C for 1 hour.

Genomic DNA was extracted with 10ml phenol (Rathburn Chemical Co.) (equilibrated in 1M then 0.1M Tris pH8) by gentle inversions. The aqueous layer was further extracted with phenol/chloroform (1:1) 2-3 times, then once more with chloroform. DNA was precipitated with 0.6 volumes 5M ammonium acetate pH 7.4 and 2 volumes of ice-cold ethanol and spooled out on a plastic loop. The DNA was rinsed in 70% ethanol and dissolved in TE at 4°C. The A_{260} was measured on a spectrophotometer (Amersham Pharmacia Biotech).

2.3.2 Digestion of genomic DNA and Southern blotting

30µg of genomic DNA was digested at 37°C for 4 hours or more with 100 units of appropriate restriction enzyme (New England Biolabs) in 1x manufacturer's buffer. The enzyme volume in the reaction was kept at less than 5% of the total. The digested DNA was precipitated overnight at 4°C (in 0.6 volumes 5M ammonium acetate pH 7.4 and 2 volumes ethanol), washed in 70% ethanol and air-dried for 10 min. 5µg DNA per

lane was resuspended in 1xDNA loading buffer, heated at 60°C for 15 min and run on a 20cm long 0.7% agarose gel until the bromophenol blue band was 1cm from the bottom. The gel was visualised under UV light to confirm digestion of the DNA. DNA was transferred and fixed onto charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech) by capillary transfer in 0.4M NaOH (Sambrook *et al.*, 1989). After transfer the membrane was rinsed in 2xSSC and air-dried.

High stringency probing of Southern blots was carried out following the method of Church and Gilbert (Church and Gilbert, 1984). Filters were prehybridised in high stringency DNA hybridisation buffer for 1-4 hours at 67°C and hybridised overnight at 67°C, in denatured probe added to high stringency DNA hybridisation buffer. Filters were washed once at room temperature (5 min) and four times for 15 min at 67°C, in high stringency washing solution. Blots were then sealed into plastic whilst damp and exposed to XOMAT film (Kodak) at –70°C for 1 hour to several days.

DNA probes were made by random-primed ^{32}P labelling (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984; Harwood, 1996). 30ng of DNA (in a final volume of 31µl with ddH_2O) was denatured by boiling for 3 min and placing immediately on ice. To the DNA was added 10µl OLB buffer, 2µl 10mg/ml BSA, 5µl labelled nucleotide (α-^{32}P dATP, >6000Ci/mmol, Redivue, Amersham Pharmacia Biotech) and 2µl of the Klenow fragment of DNA polymerase I (New England Biolabs). The reaction was incubated at 37°C for 1-2 hours. Unincorporated nucleotides were removed by spinning the mixture at 700g for 1 min through a 1cm Sephadex G50 column (BioRad) and the probe was denatured by boiling for 5 min before use in hybridisation.

2.3.3 RNA preparation and northern blotting.

2-5x10^7 *Dictyostelium* cells were placed on dry ice and lysed immediately into 500µl RNA buffer containing 1% SDS and 500µl phenol (pre-equilibrated in RNA buffer) by vortexing then shaking for 15 min at room temperature. The mixture was cooled on ice before spinning at 20 000g, 15 min, 4°C to separate aqueous and phenol layers.

350µl of the aqueous layer was removed and transferred to 500µl chloroform, the mixture was vortexed, spun as above and the lower chloroform layer discarded. RNA was precipitated for 1 hour at -20°C by adding 1ml 100% ethanol before spinning at 20 000g for 10 min, 4°C. The pellet was washed in 70% ethanol, left to air dry on

ice, and dissolved in DEPC-treated sterile ddH_2O to a final concentration of 2-10μg/μl after measuring the A_{260} on a spectrophotometer.

RNA was prepared for electrophoresis in 1x MOPS buffer, 0.2M formaldehyde, 50% v/v formamide and 10% v/v sterile RNA loading dye, denatured at 60°C for 15 min and cooled on ice. 10μg RNA per lane was loaded on a 1% agarose gel containing 1xMOPS buffer, 0.65M formaldehyde and 0.17mg/ml ethidium bromide. The gel was run in 1xMOPS buffer at 105V for 3.5 hours and visualised under UV light before equilibrating in 10xSSC and blotting onto Hybond N nylon membrane (Amersham Pharmacia Biotech) by capillary transfer in 10xSSC. The RNA was UV-crosslinked to the membrane (Stratalinker, Stratagene).

Filters were prehybridised at 42°C for 2 hours in RNA hybridisation buffer and then hybridised in denatured probe added to RNA hybridisation buffer, overnight at 42°C. Unbound probe was removed with 2xSSC/0.1%SDS washes: the first at room temperature, followed by four 15-minute washes at 67°C. The filters were given a final room temperature wash in 1xSSC before sealing in plastic and exposure to XOMAT film (Kodak) at -80°C.

2.4 Molecular biology, cloning and sub cloning

2.4.1 Agarose gel electrophoresis of DNA

Agarose gels were prepared in 0.5xTBE as described previously (Sambrook *et al.*, 1989). The percentage of the gel was determined by the size of DNA fragments to be resolved. 0.5μg/ml ethidium bromide was included in gels for visualisation of DNA under UV light. DNA was loaded in 10% v/v DNA loading buffer. 1μg of size markers was also loaded (1kb λ digest, Gibco).

2.4.2 Transformation of bacteria

Transformation was performed as described previously (Harwood, 1996). Electrocompetent bacteria (*E.coli* DH5α, Stratagene) were prepared as follows. A 10ml L-Broth overnight culture was grown from a single bacterial colony. This was diluted 1:100 and grown until logarithmic (OD_{600} of 0.5) at 37°C. The culture was placed at 4°C for 15 min and pelleted by centrifugation at 4000g, 4°C for 6 min. The cells were resuspended in an equal volume of ice-cold 0.1mM HEPES pH7.0, pelleted, resuspended in half the volume of HEPES and pelleted again. The pellet was resuspended in 20ml ice cold 10% glycerol, spun down and resuspended in 2-3ml ice

cold 10% glycerol giving a final concentration of around $3x10^{10}$ cells/ml. The electrocompetent bacteria were stored at –70°C until use.

To transform the cells, 40µl bacteria were added to a chilled 0.1cm electroporation cuvette (Flowgen) with 1-5µl of plasmid DNA in ddH_2O or TE. The cuvette was agitated and left on ice for 1 minute before electroporating in a BioRad electroporator: 1 pulse at 1.8kV, 25µF capacitance with the pulse controller at 200Ω, giving a time constant of around 4.7ms. After pulsing, 1ml of room temperature L-Broth was added to the cells, which were then incubated at 37°C for 1hour. 100µl of this suspension and 1:10 and 1:100 dilutions were spread onto LB agar plates containing the appropriate selective antibiotic (50µg/ml ampicillin unless stated otherwise). Single transformed colonies were picked using a sterile loop and inoculated into L-Broth with appropriate selection. Cultures were shaken at 37°C, 250rpm, in a tube or flask at least 5 times the volume of the culture to ensure good aeration.

2.4.3 Miniprep of bacterial plasmid DNA

DNA was prepared from overnight bacterial cultures by the rapid boiling method (Harwood, 1996). Bacteria from 1.5ml of culture were pelleted at 20 000g for 5 min in a microfuge (Hettig) and the medium removed. The pellet was vortexed and the cells lysed in 200µl STET containing 1mg/ml lysozyme in boiling water for exactly 45 seconds. Spinning the lysate (20 000g, 10 min) allowed precipitation of genomic DNA and protein as a fluffy pellet which was removed from the supernatant using a toothpick. The remaining plasmid DNA and RNA was precipitated at room temperature by addition of an equal volume of isopropanol to the supernatant and pelleting at 20 000g for 10 min. The precipitate was washed in 100µl 70% ethanol, air-dried and dissolved in 100µl TE. 10µl of the DNA (usually about 100ng) was digested with appropriate restriction enzymes and the digests treated with 1mg/ml RNAse A (previously boiled for 15 min) for 10 min at room temperature before analysis on an agarose gel.

2.4.4 Maxiprep of bacterial plasmid DNA

Large scale, pure plasmid preparations were obtained using gravity flow resin columns (Qiagen-tip 500). A 100ml culture (for high copy number plasmids, or 500ml for low copy numbers) was grown, in L-broth with selection, from a single transformed

colony overnight (16 hours)*. This ensured cells were growing logarithmically (OD_{600} of 0.5-0.7), in order to obtain high quality DNA. The cells were pelleted at 6000g for 10 min (Beckman J-25 centrifuge, JLA 10.5 rotor) and all supernatant removed by draining the inverted tube. The bacteria were resuspended homogeneously in 50mM Tris-HCl pH8.0, 10mM EDTA, 100µg/ml RNAse A, transferred to a 40ml polypropylene tube (Beckman) and lysed by adding freshly prepared 200mM NaOH, 1%SDS for 5 min with mixing by gentle inversions. Proteins, genomic DNA and cell debris were precipitated by adding chilled 3M potassium acetate pH5.5, mixing gently and leaving on ice for 15 min. The precipitate was spun out at 20 000g for 10 min (Beckman J-25 centrifuge, JA 25.5 rotor). The supernatant, containing plasmid DNA, was filtered through sterile glass wool into a Qiagen-tip column previously equilibrated with 30ml solution containing 750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% TritonX-100. Plasmid DNA remained bound to the resin and was washed twice in 30ml wash buffer (1M NaCl, 50mM MOPS pH7.0, 50% isopropanol before elution with 15ml of 1.25M NaCl, 50mM Tris pH8.5, 15% isopropanol into a 30ml Corex tube (Corning). DNA was precipitated by immediately adding 0.7 volumes of room temperature isopropanol and centrifuging at 20 000g, 30 min, 4°C. The DNA pellet was washed in 5ml 70% ethanol, recentrifuged for 5 min, air-dried and resuspended in 500µl of TE. The A_{260} was measured on a spectrophotometer to determine the yield.

2.4.5 Restriction enzyme digestion and analysis of plasmid DNA

0.1-1µg of DNA was digested with at least a 5-fold excess (1µl enzyme per 20µl digest) of appropriate restriction enzyme (New England Biolabs) in 1x manufacturer's buffer for 1 hour at 37°C. For analysis, half the digest was added to 1µl 10x DNA loading buffer and DNA fragments separated on TBE agarose gels.

2.4.6 Gel purification of DNA fragments

DNA fragments were separated on 0.8% TBE/agarose gels and the relevant bands excised. Gel slices were melted in >3 volumes of 6M NaI, 0.12M Na_2SO_3, 0.3M Na acetate for 15 min at 55°C. 5µl of silica bead slurry (Sigma) in ddH_2O was added per 10µg of DNA to be recovered and the mixture incubated at room temperature for 30 min. The glass milk was pelleted (20 000g, 5 min) and resuspended in 1ml ethanol wash (50% ethanol, 100mM NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA) before pelleting, washing again and air-drying. DNA was eluted from the glass milk in two

* Glycerol stocks of transformed bacteria were prepared by adding 200µl of 100% glycerol to 800µl of fresh bacterial culture. Stocks were stored at –80°C.

10µl batches of TE by heating to 55°C for 10 min. Elutions were pooled and a 4µl sample run out on a 0.8% agarose gel to determine the yield.

2.4.7 Sequencing and analysis of DNA

Sequences were obtained from maxiprep DNA or from PCR products. PCR fragments were purified away from the PCR reaction using spin columns (S400, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Sequencing was carried out by cycle sequencing using fluorescent dye-labelled terminating dideoxynucleotide triphosphates (ABI Prism™ kits, dye terminator or dRhodamine, Perkin Elmer Biosystems) according to the manufacturer's instructions. Briefly, 500ng plasmid DNA or 50ng PCR product was added to 3.2pmol sequencing primer and 8µl Terminator Ready Reaction mix(in a total volume of 20µl. Cycle sequencing was carried out in a Perkin Elmer PTC-100 thermal cycler: (24 cycles of 95°C for 30s, 50°C 15s, 60°C 4 min). Sequenced DNA was purified away from the reaction mix using a spin column (G50, Amersham Pharmacia Biotech), according to the manufacturer's instructions, and lyophilised. All dRhodamine and some dye terminator reactions were run on sequencing gels by Oswel Sequencing Service at Southampton University, U.K. Some DNA was sequenced in full by MWG Biotech using a LiCOR machine. Other reactions were run on an ABI Prism™ 377 sequencer as follows.

Sequencing gel plates were washed in ddH_2O, dried, and assembled with spacers for gel pouring. The acrylamide gel mix (4.25% (w/v) acrylamide/bisacrylamide (19:1), 6M urea, 1xTBE sequencing mix; Anachem) was filtered through a 0.22µm filter (Millipore) and left to degas at room temperature for 5 min. 83µl of fresh 30% APS and 35µl TEMED were added and mixed gently. The gel mix was loaded into the plates from a syringe, ensuring no air bubbles were present, the gel comb inserted upside down, and the gel left to polymerise for two hours. The comb was removed and any loose pieces of acrylamide were rinsed from the top of the gel with ddH_2O. The comb was replaced with the teeth just touching the top of the gel and the gel was assembled on the sequencing machine with running buffer (1xTBE, National Diagnostics) in the upper and lower chambers. Dried sequencing reactions were resuspended in 4µl sequencing gel loading dye and denatured at 95°C for 5 min before placing on ice. The gel was run using ABI Prism™ software. Briefly, the assembled gel was scanned to check for inherent fluorescence before prerunning until the temperature was 51°C. 1.5µl sequencing samples were loaded in alternate (washed) wells across the gel and run

into the gel for 5 min before loading the remaining samples. The outermost wells were loaded with buffer alone to ensure even running of the samples nearest the edges of the gel and the gel was run for 7 hours. After running, the gel image was tracked and sample data extracted according to the manufacturer's instructions. The raw sequencing data was analysed using EditView (ABI Prism™) and DNAStar™ software.

2.4.8 cDNA library screening

2.4.8(i) Expression library screening

A λZAPII (Alting-Mees and Short, 1989) cDNA library for 6-8 hours of development (Stratagene; provided by Dr E. Snaar-Jagalska, University of Leiden, The Netherlands) was initially used as an expression library for probing with anti-HPGN antiserum.

The titre of the library was determined by serial dilution into sterile λSM and plating onto 10cm λ library bottom agar plates (in 3ml λ library top agarose, see below), incubation overnight at 37°C and counting the plaques formed. $2x10^5$ plaque forming units (pfu) were used per 20cm plate (Nunc) for screening: 5 plates were used for a primary screen.

A 50ml overnight culture of XL-1 blue host bacteria was grown in L-Broth supplemented with 0.2% maltose, 10mM $MgSO_4$, 10μg/ml tetracycline. Bacteria were harvested and resuspended in 20ml sterile 10mM $MgSO_4$: these bacteria could be stored at 4°C for a few days before use in top agarose.

Screening plates were prepared as follows: λ library bottom agar was poured (250ml in a 20cm plate) and allowed to set and dry. The bacteriophage ($2x10^5$ pfu/plate) were incubated with 3ml of host bacteria in $MgSO_4$ for 30 min and plated by mixing with 50ml λ library top agarose at 50°C and pouring carefully and quickly across the bottom agar. Plates were incubated, inverted, for 8-12 hours until "pinprick" plaques could be seen on the bacterial lawn. At this stage protein expression was induced by overlaying IPTG-soaked filters on the plaques (Hybond C-extra supported nitrocellulose (Amersham Pharmacia Biotech) soaked in 10mM IPTG for 5 min and air-dried). Plates were incubated at 37°C for 2-4 hours or overnight. The orientation of the filters was marked by making holes in the corners of the filters and the agar.

Protein-coated filters did not need fixing and were washed twice in PBS and blocked in 5% milk, 0.1% Tween in PBS for 1 hour. Five 10-min washes were then carried out in PBST and filters were incubated with primary antiserum, anti-HPGN, in

PBST (1:5000) for 4 hours at room temperature. Four 10-minute washes were performed before incubation in secondary antibody for 1 hour at room temperature. After four more washes filters were treated with ECL reagents and exposed to BioMax film as for western blotting, exposures were for 1 hour.

Positive colonies were picked by aligning the black "spots" on the autoradiograph with the library plate and excising the plaque and surrounding area of the agar with the open end of a 1ml Gilson pipette tip. DNA from agar plugs was eluted by storing the plug in λSM containing a drop of chloroform at 4°C overnight. At this stage, colony DNA was not pure (clonal) and second and third rounds of screening were carried out on 9cm plates (with 3ml top agarose and 300μl host bacteria) using 100μl of 10^{-4}, 10^{-5}, and 10^{-6} dilutions of the picked, eluted colonies. This rendered the colony density low enough to pick a single, pure positive plaque. A negative primary colony was also picked and rescreened in the same way as a control for background, non-specific antibody binding to high concentrations of phage-expressed proteins.

2.4.8(ii) Library screening with DNA oligomers

The λZAPII library described above and a λZAPII slug library (from Dr M. Fukuzawa, MRC-LMCB, London, UK) were used to probe for cDNA. Titreing and plating out (2 plates, $2x10^5$pfu/plate) was performed as for expression library screening. 20cmx20cm screening plates (Nunc) were incubated, inverted, for 10-12 hours until clear plaques about 1mm across could be seen in the bacterial lawn. At this stage charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech) was placed carefully on the plates to transfer colony DNA, and the orientation of the filter was marked as before. The membrane was lifted after 1 minute, and the colony plate returned to 37°C for a further 3 hours, then a second (duplicate) lift was carried out for 3 min.

DNA was denatured by placing the filter, DNA side up, onto a pad of 3MM paper (Whatman, 2 sheets) soaked in 1.5M NaCl, 0.5M NaOH for 7 min. Filters were then neutralised by placing on a pad soaked in 1.5M NaCl, 0.5M Tris-HCl pH7.2, 1mM EDTA for 5 min, washed for 30 seconds in 2xSSC and air-dried. DNA was then fixed by placing the filters on a pad of 0.4M NaOH for 20 min before further rinsing in 5xSSC and air-drying. Filters were probed with end-labelled PCR primers (Section 2.4.8(iii)) or with random-primed radiolabelled *aar* full-length cDNA (previously digested away from pBluescript with *Sal*I and *Not*I restriction enzymes and gene

cleaned) using high stringency hybridisation (section 2.3.2) but with 1-hour washes in 1-litre volumes to eliminate background.

After 3 rounds of screening positive clones were excised (section 2.4.9) or the inserts amplified by PCR (section 2.4.10) using universal T3 and T7 primers and the PCR fragment sequenced (section 2.4.7).

2.4.8(iii) End labelling of short oligonucleotides and probing cDNA library filters

250pmol of a mixture of degenerate primers were end-labelled with γ-^{32}PdATP as follows. To the oligonucleotide was added 7.5µl 10x T4 exchange buffer (New England Biolabs), 66pmol (~200µCi) (>5000Ci/mmol, Amersham Pharmacia Biotech) γ-^{32}PdATP, 5µl T4 polynucleotide kinase (New England Biolabs) in a final volume of 75µl in ddH_2O. The labelling reaction was incubated for 1 hour at 37°C and the probe purified on a G50 spin column (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Primary library filters (Section 2.4.8(ii)) were incubated for 4 hours at 45°C (~5°C below the predicted Tm of the oligonucleotides) in 0.1ml/cm^2 low stringency DNA prehybridisation solution. The probe was bound to the filters in low stringency hybridisation solution overnight at 50°C. Filters were washed three times at 50°C in 2xSSC and once in 1xSSC (1 hour each, until no more unincorporated oligonucleotide was present in the wash buffer) and exposed to XOMAT film (Kodak) at –80°C. Positive plaques were picked as before and these non-clonal λ pools were subjected to PCR with T3 and T7 primers (Section 2.4.10) to amplify the inserts in each pool. The PCR products were separated on a 0.8% agarose gel, blotted onto nitrocellulose in 0.4M NaOH (section 2.3.2) and probed with the oligonucleotide mix. Positive λ pools were replated onto agar at various dilutions in order to pick individual plaques. 20 plaque inserts were reamplified by PCR, separated on 0.8% agarose, blotted and reprobed. Positive PCR products were prepared for sequencing (section 2.4.7).

2.4.9 Excision of positive clones

Positive *aar* cDNA clones were excised from a host bacterial genome as circularised plasmids (pBluescriptIISK+/-) by incubation of host bacteria with helper phage (Stratagene rapid excision kit) as follows. 5ml cultures of excision (XPORT) and plasmid growth (XLOLR) bacteria were grown to an OD_{600} of 2.0 in NZY broth and placed at room temperature without agitation for 30 min. ~10-1000pfu of the positive λ

clone (0.1 –3µl) was mixed with 50µl XPORT culture, 10µl 704 helper phage, and 5µl XLOLR culture. To this was added 3ml NZY top agarose at 50°C and the whole mixture was poured over 10cm NZY agar plates and incubated at 37°C overnight. Turbid "excision plaques" were formed, which contain excised, packaged phagemid DNA from the coreplication of λ phage and helper phage in XPORT. These plaques also contain λ phage particles, helper phage particles, lysed XPORT cells and XLOLR cells infected with phagemid, but resistant to infection by λ phage or helper phage. These excision plaques were picked using a sterile loop and grown overnight in L-Broth with 50µg/ml tetracycline and 50µg/ml ampicillin to select for growth of only XLOLR bacteria (tet^R) containing pBluescript phagemids with the insert of interest (amp^R). Plasmids were purified from the cultures by miniprep and digested with appropriate restriction enzymes to diagnose positive clones. Positive miniprep plasmid DNA was transformed into DH5α bacteria and grown up for maxipreps to produce sequencing quality DNA.

2.4.10 PCR

Polymerase chain reaction (PCR) to amplify specific DNA fragments was carried out on a Perkin Elmer PTC-100 thermal cycler. 50µl reactions were set up in thin-walled 200µl tubes (Radleys) or multiwell plates (Perkin Elmer), as follows. 5µl λ DNA (boiled in 5mM EDTA before use) or 0.1µg *aar* DNA (or ddH_2O control) was mixed with 2µmol each primer (or 1 primer plus ddH_2O for controls), 200µM each of dATP, dCTP, dTTP and dGTP (Pharmacia), 1x Pfu buffer (Stratagene), 2.5 units of *Taq* polymerase and 2.5 units of recombinant *Pfu* polymerase (Stratagene).

For amplification of the λ DNA described in section 2.4.8 (iii), the DNA was denatured at 95°C for 1 minute then subjected to 30 cycles of 95°C for 15s, 55°C for 30s, 72°C for 2 min followed by a final extension step at 72°C for 10 min before storage at 4°C. These conditions were also used for diagnostic PCR of *aar* DNA where the expected product size was 1.2kb or less. For amplification of larger PCR products extension times of 6 min were used in the cycles. For amplification of *aar* genomic DNA the annealing temperature was lowered to 50°C and annealing performed for 1 minute. For generating the inserts for pTrcHisB (section 2.5) the PCR reaction was supplemented with a further 2mM $MgSO_4$, bringing the total Mg^{2+} content of the reaction to 4mM.

2.5 Aardvark fusion protein preparation and antibody generation

A His-tagged fusion protein containing the last 5 Armadillo repeats of Aardvark was used for immunisation. A PCR primer was designed to introduce a *Xho*I site into the *aar* cDNA sequence at the junction between repeats 5 and 6. This was used on the *aar* cDNA with the T7 primer to amplify the 3' end of *aar* (Section 2.4.10). The resulting PCR fragment was cut with *Xho*I and *Pst*I and ligated into the pTrcHisB vector (Invitrogen) also cut with *Xho*I and *Pst*I. This vector adds an amino-terminal polyhistidine (6xHis) tag and antibody recognition epitope to the in-frame translated PCR product. The ligation product DNA was transformed into DH5α.

To induce protein expression 1l of bacterial culture was grown at 37°C in L-Broth supplemented with 100μg/ml carbenicillin to an OD_{550} of 0.6 before adding 1mM IPTG and inducing protein expression at 22°C for 1 hour. Cells were harvested (6000g, 5 min, 4°C) and lysed in 80ml bacterial native lysis buffer supplemented with 1mM AEBSF, 10mg/ml aprotinin, 0.75mg/ml lysozyme for 20 min at room temperature. DNA was disrupted by sonication (three 5 second blasts on ice) and insoluble material pelleted (10 000 g, 20 min, 4°C). The 6xHis-tagged protein from 20ml clarified supernatant was bound in batch to 1ml Talon™ (Clontech) metal affinity resin for 20 min at room temperature and loaded onto a 10ml gravity flow column (BioRad). The column was washed with 10ml bacterial native lysis buffer pH6, 10ml native lysis buffer pH5 and 20ml native lysis buffer pH8 containing 10mM imidazole. 6xHis-tagged protein was eluted with 4ml native lysis buffer pH8 containing 75mM imidazole, and 250μl fractions were collected. The two peak protein fractions (as analysed by Coomassie-stained PAGE gels) were pooled, neutralised and mixed 1:1 with adjuvant; this was sufficient for one immunisation.

One New Zealand White male rabbit (whose preimmune serum had previously been shown to give no background by immunofluorescence on *Dictyostelium* cells, or on western blots of whole cell extracts) was immunised four times, at 14 day intervals. The first immunisation contained complete adjuvant (H37Ra Sigma); the three subsequent immunisations used incomplete adjuvant (Sigma).

2.6 Rapid freezing and electron microscopy of *Dictyostelium* culminants

2.6.1 Preparation of culminants for scanning electron microscopy (SEM)

Wild type (AX2) and mutant (*aar*$^-$) cells from logarithmically growing axenic cultures were plated on nitrocellulose filters (Whatman) at a density of $5x10^7$ cells per

filter. For synchronous development to mid-culminant AX2 cells were incubated for 18 hours and *aar*⁻ for 23 hours, at 22°C. Culminants were rapidly frozen by placing the filters flat on a polished copper block pre-cooled in liquid nitrogen. The structures were then dessicated by freeze drying for 48 hours in a Kinney vacuum evaporator and coated with 100nm gold/palladium in a HummerV sputter coater. Filters were mounted with double-sided tape on aluminium stubs and earthed with silver paint for examination by SEM.

2.6.2 Scanning electron microscopy

Culminants were examined on a Hitachi S-570 scanning electron microscope at 12kV accelerating voltage. Images were photographed on Kodak TMAX100 35mm film and developed and printed by hand.

2.6.3 Preparation of culminants for transmission electron microscopy

Wild type and mutant cells were developed on filters and fixed by ultra-rapid freezing, plunging the filters (culminant first) into liquid propane cooled by liquid nitrogen, in a plunging device designed by Dr. H. Mollenhauer (personal communication to M.J. Grimson). Frozen structures were stored in liquid nitrogen and freeze substitution was carried out by placing the culminants in 10ml of precooled acetone (for immuno-EM) or acetone/1% osmium tetroxide (to examine ultrastructure) at –80°C in a massive brass block (Blockmeister™, CheapScience inc.). Blocks were returned to room temperature slowly over several days by moving them to subsequently higher temperatures (-80°C, -40°C, -20°C, 4°C, room temperature). Before sectioning, the culminants were infiltrated with a dilution series (3:1, 1:1, 1:3) of acetone:Spurr's epoxy resin* over three days and then infiltrated with Spurr's resin alone between two glass slides and hardened overnight at 70°C to embed the structures. Embedded structures were examined at 20x magnification under an Olympus BX50 fluorescence microscope with DIC optics; images were captured using an Optronics TEC470 video camera and NIHimage software. 0.5μm-0.08μm sections were prepared from embedded structures using a Sorvall MT2B microtome. Thick (0.5μm) sections were stained with toluidine blue for examination by light microscopy using an Olympus BX50 microscope (20x magnification) and photographed on TMAX100 35mm film (Kodak).

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* (Electron Microscopy Sciences Fort, Washington, PA)

2.6.4 Transmission electron microscopy (TEM)

Thin sections were floated onto gold specimen grids (BAL-TEC) and examined at 75kV in a Hitachi HU-11E transmission electron microscope. Images were photographed on Kodak 4489 film and developed and printed by hand.

2.7 Cell biology methods

2.7.1 Stalk and spore assays

Cells were plated at low density in monolayer cultures (Harwood *et al.*, 1995). For stalk cell induction, cells were plated at $2.9x10^4/cm^2$ in 6cm or 3cm diameter tissue culture dishes (Falcon) and incubated for 24 hours at 22°C in stalk medium supplemented with 5mM cAMP. Cells were washed three times in stalk medium and incubated for a further 24 hours with 0.1μm DIF with or without 5mM cAMP, in stalk medium. Vacuolated stalk cells were counted on a Zeiss inverted microscope and expressed as a percentage of the total cell count. Spore cells were induced by plating amoebae at $1.5x10^3$ cells/cm^2 in spore medium supplemented with 15mM 8-BrcAMP (Kay, 1989) and incubating for 48 hours at 22°C. Elliptical, refractile spore cells were counted and expressed as a percentage of the total cell count.

Spore viability was assayed by scraping mature fruiting bodies from an SM/*Klebsiella* plate into cold horse serum and diluting ten-fold into 0.3% Nonidet P-40 (NP40) in KK_2. Fruiting bodies were incubated for 30 min to lyse non-spore material before spinning at 20 000g for 10 min to pellet the spores which were then washed four times in KK_2, counted and plated at either 50 or 500 per SM/*Klebsiella* plate. After a few days, colonies formed from viable spores could be counted and expressed as a percentage of the original number of spores plated. 10^8 vegetative cells were treated with detergent in the same way and plated, as a negative control.

2.7.2 ß-galactosidase staining of whole fruiting bodies

Staining was performed similarly to previous experiments (Dingermann *et al.*, 1989). Briefly, transformed wild type (AX2) and *aar*$^-$ cells were developed on nitrocellulose filters and fixed in 1% v/v glutaraldehyde in Z-buffer (KK_2/1mM $MgCl_2$) for 10 min. Fixed structures were washed three times in Z-buffer before incubation in X-gal staining solution for 1-10 hours at 37°C in a moist chamber to allow the blue stain to form. Stained structures were photographed at 50x magnification on the filters using a dissecting microscope (Wild M8 with MPS11 camera). Alternatively, structures were placed onto glass slides in slide mountant, with a coverslip, and photographed at high

magnification (200-400x) on a Zeiss Axioskop fluorescence microscope with MC100 camera, using integrated exposure times and Kodak Ektachrome 160T 35mm slide film.

2.7.3 Methylene blue staining of developing cells

Amoebae were taken from SM/*Klebsiella* plates 6 hours after clearing and resuspended in 1mg/ml methylene blue (Fisher Scientific) for 15 seconds to stain the developing prestalk cells (Sternfeld, 1992). Stained cells were washed in KK_2, replated on water agar and exposed to unidirectional light in humid conditions for 24 hours, allowing slugs to form and migrate. Culmination of slugs was induced by removing the lids from the plates and placing in the light. Structures were photographed at 50x magnification under a dissecting microscope (Wild) on Ektachrome 160T 35mm slide film (Kodak).

2.7.4 Neutral red staining of developing cells

Logarithmically growing axenic cells were pelleted and washed twice in KK_2. To stain the cells 5 drops of neutral red (0.2mg/ml in ddH_2O) were added to the pellet, which was vortexed for 15-30 seconds. The pellet was immediately washed twice more in KK_2. Cells were plated on 1.8% KK_2 agar, allowed to develop and photographed under a dissecting microscope as in section 2.7.3.

2.7.5 Tinopal staining of fruiting bodies

To visualise the presence of cellulose in the walls of mature stalks and spores cells were developed to the culminant stage on 1% KK2 agar containing 0.1% w/v Tinopal LPW (a gift from Dr. L. Blanton), a UV-fluorescent dye which binds to polysaccharides. Developed structures were placed on a slide in mountant with a coverslip and visualised under UV light on a Zeiss Axioskop fluorescence microscope as before.

2.8 Recipes and reagents

Standard laboratory chemicals were from BDH. Other chemicals were from Sigma Chemical Company unless otherwise stated.

2.8.1 Media

Axenic medium	1.43% peptone (Oxoid, L34)
	0.72% yeast extract (Oxoid L21)
	3.6mM Na_2HPO_4, 3mM KH_2PO_4
	30% glucose,
	0.5mg/ml vitamin B12, 1mg/ml folic acid pH9.0
	Final pH 6.4 (sterile)
HEPES-HL5	20mM HEPES pH 7.05
	0.5% yeast extract (Oxoid L21)
	1% peptone (Oxoid L34)
	1% glucose
HBS	270mM NaCl
	10mM KCl
	1.2mM Na_2HPO_4
	40mM HEPES pH7.05
	0.2% glucose
KK_2	15.5mM KH_2PO_4
	3.8mM K_2HPO_4
	Final pH 6.2
L-Broth (sterile)	1% bactotryptone (Difco)
	0.5% bacto-yeast extract (Difco)
	17mM NaCl
	Final pH 7.0
LB agar	L-broth
	1.5% Bactoagar (Difco)

NZY broth

1% NZYCM (Difco)

0.5% bacto-yeast extract (Difco)

8.6 mm NaCl

0.8mM $MgSO_4.7H_20$

Final pH 7.0

NZY agar

NZY broth

1.5% bactoagar (Difco)

NZY top agar

NZY broth

0.7% agarose (Boehringer Mannheim)

SM (Sussman's medium)

1% glucose

1% peptone (Oxoid L34)

0.1% yeast extract (Oxoid L21)

2% agar (Difco)

4mM $MgSO_4$

4mM KH_2PO_4

6mM K_2HPO_4

2.8.2 Molecular Biology

50x Denhardt's solution

1% BSA (FractionV)

1% Ficoll

1% Polyvinylpyrollidine

DNA loading buffer

0.25% bromophenol blue

0.5% xylene cyanol

15% Ficoll-400

Genomic DNA lysis buffer

20mM Tris-HCl pH7.5

5mM $MgCl_2$

0.32M sucrose

0.02% sodium azide

1% Triton X-100

Genomic DNA digestion buffer

10mM Tris-HCl pH 7.5

5mM EDTA

0.7% SDS

High stringency DNA hybridisation buffer

500mM Na_2HPO_4/NaH_2PO_4 pH 7.2

7% SDS

High stringency DNA wash buffer

40mM Na_2HPO_4/NaH_2PO_4 pH 7.2

1% SDS

Low stringency DNA prehybridisation solution

6xSSC

5x Denhardt's solution

0.1mg/ml boiled, sheared herring sperm DNA

0.05% sodium pyrophosphate

0.5% SDS

Low stringency DNA hybridisation buffer

6xSSC

1x Denhardt's solution

0.05% sodium pyrophosphate.

λ SM	100mM NaCl 13mM $MgSO_4.2H_20$ 50mM Tris-HCl pH7.5 0.01% gelatin
λ library bottom agar	LB agar 10mM $MgSO_4$ 0.2% maltose 10μg/ml tetracycline
λ library top agarose	L broth 0.7% agarose (Boehringer Mannheim) 0.2% maltose 10mM $MgSO_4$
MOPS buffer	20mM MOPS pH7.0 5mM Na Acetate 1mM EDTA
OLB Buffer	Solutions A:B:C at 100:250:150 stored at –20°C. Solution A: 125mM Tris-HCl pH8, 125mM MgCl2, 1.8% ß-mercaptoethanol, 0.5% each of dCTP, dTTP, dGTP (nucleotides stored as a 0.1M stock in 3mM Tris-HCl, 0.2mM EDTA pH7). Solution B: 2M HEPES pH adjusted to 6.6 with 4M NaOH Solution C: Random hexadeoxyribonucleotides (Pharmacia) at 4.5mg/ml in TE pH8
RNA buffer	100mM Tris-HCl, pH 7.4 200mM NaCl, 20mM EDTA

RNA hybridisation buffer

43% v/v formamide
5x SSC
10x Denhardt's solution
10mM Na_2HPO_4/NaH_2PO_4 pH 6.8
200mg/ml denatured, sheared herring sperm DNA
0.1% SDS

RNA loading dye

50% v/v glycerol
1mM EDTA
0.4% w/v bromophenol blue
0.4% w/v xylene cyanol

Sequencing gel loading dye

5:1 deionized formamide: 25mM EDTA pH8.0
50mg/ml blue dextran

SSC

150mM NaCl
15mM Na_3citrate

STET

50mM Tris-HCl pH8
50mM EDTA
8% sucrose
5% Triton-X 100

0.5x TBE

45mM Tris-HCl
45mM Boric acid
1mM EDTA

TE

10mM Tris-HCl pH 7.4
1mM EDTA

2.8.3 Biochemistry

Bacterial native lysis buffer

10mM Tris-HCl
50mM Na phosphate
200mM NaCl pH8

Buffer A

50mM Tris-HCl pH7.5
50mM NaCl

CLAP 1000x

5mg/ml Chymostatin
10mg/ml Leupeptin
5mg/ml Antipain
5mg/ml Pepstatin A
dissolved in DMSO

Coomassie destain

25% methanol
16% acetic acid

Coomassie stain

0.25% Coomassie Brilliant Blue
10% acetic acid
45% methanol

Laemmli buffer

10% glycerol
100mM DTT
2% SDS
50mM Tris-HCl pH6.8
0.1% bromophenol blue

PBS

137mM NaCl
2.68mM KCl
7.98mM Na_2HPO_4
1.47mM KH_2PO_4
Final pH 7.2

2.8.4 Cell Biology

Slide mountant	Add 140mM NaCl 0.04% Na azide, 25% w/v polyvinyl alcohol and 2.5% DABCO anti-fading agent to 160ml PBS and stir overnight. Add 80ml glycerol and stir overnight. To clear, centrifuge at 5500g, 15 min, 4°C. Store at 4°C.
Spore medium	10mM MES pH 6.2 20mM NaCl, 20mM KCl 1mM $MgCl_2$ 1mM $CaCl_2$ 200µg/ml streptomycin, 20µg/ml tetracycline
Stalk medium	10mM MES pH 6.2 2mM NaCl 10mM KCl, 1mM $CaCl_2$ 200µg/ml streptomycin, 20µg/ml tetracycline
X-gal staining solution	Z-buffer 2.5mM $K_4Fe(CN)_6$ 2.5mM $K_3Fe(CN)_6$ 0.1% X-gal
Z-buffer	KK_2 1mM $MgCl_2$

2.9 List of abbreviations

aar, Aar, *aar*$^-$	Aardvark gene, protein product and knock-out
AEBSF	4-(2-Aminoethyl)benzenesulphonyl Fluoride
amp^R	Ampicillin resistant
APS	Ammonium persulphate
bp	base pairs
8-BrcAMP	8-Bromo cAMP

BSA	Bovine serum albumin (Fraction V)
°C	degrees Celsius
cAMP	Adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
Ci	Curie
CLAP	Chymostatin, Leupeptin, Antipain, Pepstatin protease inhibitors
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytosine 5'-triphoshate
ddH_2O	Double distilled water (Millipore)
DEPC	Diethyl pyrocarbonate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DIF	Differentiation Inducing Factor
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
dTTP	Thymidine 5'-triphosphate
ECL	Enhanced chemiluminescence
EDTA	Disodium Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis(ß-aminoethylether)-N,N,N'N'-tetraacetic acid
F	Farad
g	gram
GST	Glutathione-S-Transferase
HBS	HEPES-buffered saline
HEPES	N-2 Hydroxyethylpiperazine-N'2-ethanesulphonic acid
his-tag	6-histidine epitope tag
hrp	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl ß-D-thiogalactoside
kb	kilobase
kDa	kiloDalton
l	litre
M	molar
m	metre
m (prefix)	milli (10^{-3})
µ	micro (10^{-6})

min	minutes
mol	moles
MOPS	3-(N-Morpholino)propanesulphonid acid
mRNA	Messenger RNA
n	nano (10^{-9})
OD_x	Optical density at x nm
p	pico (10^{-12})
^{32}P	Radioactive phosphorous
PBS	Phosphate-buffered saline
PBST	PBS/0.1% Tween-20
pfu	Plaque-forming units
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SSC	Standard sodium citrate
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
TEM	Transmission electron microscopy
TEMED	N'N'N'N'Tetramethylethylenediamine
tet^R	Tetracycline resistant
TRITC	Tetramethylrhodamine B Isothiocyanate
UV	Ultraviolet
V	Volt
v/v	volume for volume
w/v	weight for volume

Chapter 3

Evidence for Armadillo/ß-catenin and plakoglobin related proteins in *Dictyostelium*

3.1 Introduction

The first goal of my project was to search for Armadillo/ß-catenin/plakoglobin homologues in *Dictyostelium discoideum*. *Dictyostelium* whole cell extracts were screened by western blotting with a panel of available antisera raised to Armadillo, ß-catenin or plakoglobin. It is well established that many antisera to ß-catenin and plakoglobin recognise their cognate proteins from a variety of different species ((Butz *et al.*, 1992; Cowin *et al.*, 1986); A. Magee pers. commun.). Importantly, antisera to vertebrate ß-catenin and plakoglobin recognise these two distinct proteins (92kDa and 83kDa respectively) without cross-reaction, despite their sequence similarity ((Butz *et al.*, 1992), A. Magee pers. commun.) and antisera to *Drosophila* Armadillo (Arm) cross react with vertebrate ß-catenin but not plakoglobin (Knudsen and Wheelock, 1992; Peifer *et al.*, 1992). Such antisera might therefore detect related proteins in *Dictyostelium* and be able to distinguish between Arm/ß-catenin and plakoglobin homologues in the same way.

Screening whole cell extracts from different stages of the *Dictyostelium* developmental cycle had the advantage of allowing me to determine rapidly whether the expression of any putative Arm/ß-catenin/plakoglobin homologues varied during development, as might be expected for proteins involved in specific signalling events and/or regulated cell-cell adhesion. In addition, these findings were extended by examining the expression of such proteins in the *gskA*$^-$ mutant (Harwood *et al.*, 1995), to determine whether *Dictyostelium* Arm/ß-catenin/plakoglobin levels were potentially regulated by a gsk3ß homologue, as observed in metazoa.

3.2 Antisera to Armadillo/ß-catenin and plakoglobin recognise distinct *Dictyostelium* proteins.

Wild type (AX2) cells were developed on non-nutrient agar and harvested at the first finger/slug stage. This developmental stage was chosen because well-established cell-cell contacts are present. Whole cell extracts (equivalent of 10^6 cells per lane) were separated by SDS-PAGE and transferred to nitrocellulose for western blotting. A positive control (10^5 cell equivalents per lane of human keratinocyte whole cell extract (from Dr. V. Braga, MRC LMCB, U.K.) was also tested. Blots were probed with a number of antisera.

Figure 3.1a shows that anti-HPGN, a rabbit antiserum raised to the N-terminus of human plakoglobin (including four Arm repeats) recognises a single protein of

approximately 70kDa in *Dictyostelium*. In the keratinocyte positive control, anti-HPGN detects a single protein of approximately 80kDa, the size expected for vertebrate plakoglobin (Cowin *et al.*, 1986). The binding of anti-HPGN to the *Dictyostelium* protein is specific: it can be competed by the plakoglobin-GST fusion protein to which it was raised. No cross-reacting proteins were detected with anti-HPGN pre-immune serum or with secondary antibody alone. To test whether anti-plakoglobin antisera raised in other species could detect the same *Dictyostelium* protein that was recognised by anti-HPGN, cell extracts were probed with two antisera raised to *Xenopus* plakoglobin. Anti-XPGN, raised to the N-terminus of *Xenopus* plakoglobin (not including the Arm repeats), also recognised a 70kDa protein in *Dictyostelium* (figure 3.1a). However, anti-XPGC, raised to the C-terminus of *Xenopus* plakoglobin (outside the Arm repeat region) did not recognise any protein in *Dictyostelium* although it detected an 80kDa protein in keratinocytes (figure 3.1a).

To determine the size of the *Dictyostelium* ~70kDa protein accurately, its mobility was tested against known molecular weight standards (BioRad, Kaleidoscope prestained markers) on SDS-PAGE using a 14.5cm long, 7.5% resolving gel. The standards resolved in a linear fashion as shown when their sizes were plotted logarithmically; the proteins to be tested also ran within the linear range under these conditions (figure 3.2). The size of the *Dictyostelium* protein recognised by anti-HPGN and anti-XPGN was 69kDa, that of human plakoglobin 83kDa (figure 3.2).

These results indicate that the 69kDa protein is genuinely related to vertebrate plakoglobin; henceforth this protein will be referred to as Ddplako. Ddplako is specifically recognised by antisera to the N-terminus of plakoglobin from two different species. Arm/ß-catenin and plakoglobin have a lower degree of homology in their N- and C-termini than in their Arm repeat regions (Butz *et al.*, 1992; Peifer *et al.*, 1992). Ddplako appears to have a "plakoglobin-like" N-terminus (rather than simply being an Arm repeat containing protein) as it is recognised by anti-XPGN. Ddplako is 14kDa smaller than vertebrate plakoglobin. Since an antiserum to the C-terminus of *Xenopus* plakoglobin does not recognise Ddplako, it appears that this region of the protein is not conserved and may even be missing altogether.

Screening of *Dictyostelium* first finger/slug whole cell extracts was repeated with an antiserum to human ß-catenin, VB2, raised to the C terminal peptide (Braga *et al.*, 1995). This antiserum recognises a major protein of approximately 90kDa and another of approximately 130kDa (figure 3.1b). A small 40kDa band is also sometimes seen (figure 3.1b). Only one 90kDa protein is detected in keratinocytes. All three

Dictyostelium proteins recognised by VB2 can be competed out by prior immunoabsorption of the antiserum with the peptide to which it was raised (figure 3.1b). In addition, three proteins with the same mobilities as those recognised by VB2 are detected with an antiserum raised to the C-terminal 40kDa of *Drosophila* Arm (figure 3.1b). The sizes of the two larger *Dictyostelium* proteins were determined accurately as 92kDa and 130kDa using the conditions described for Ddplako above (figure 3.2). The 92kDa *Dictyostelium* protein is the same size as vertebrate ß-catenin (figure 3.2; (Ozawa *et al.*, 1989) and similar in size to Arm, which is 92kDa when *in vitro* translated and 99-103kDa in cell extracts (Riggleman *et al.*, 1990). However all three *Dictyostelium* proteins are related, as shown by the immunoabsorption experiment. The 130kDa protein could be a distinct Arm/ß-catenin homologue, or a modified form of the 92kDa protein. The 40kDa protein could be a degradation product of Ddarm, as certain forms of ß-catenin have a short half-life (Munemitsu *et al.*, 1995; Papkoff *et al.*, 1996). Henceforth these three proteins will be collectively referred to as Ddarm.

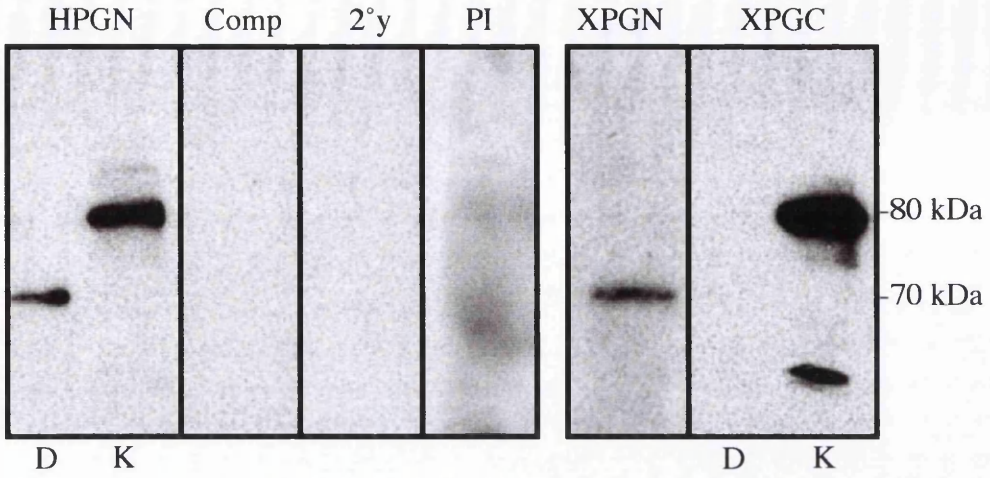

Figure 3.1a *Dictyostelium* first finger/slug whole cell extracts probed with antisera to plakoglobin.
An antiserum raised to the N-terminus of human plakoglobin cross reacts with a ~70kDa protein in *Dictyostelium* (HPGN, lane D). Human plakoglobin runs at 80kDa (HPGN, lane K, keratinocyte extract). The *Dictyostelium* signal is specific: it can be competed out by preabsorbing the antiserum with HPGN protein (Comp). Secondary antibody alone (2°y) and preimmune serum (PI) do not cross react with *Dictyostelium* cell extract. An antiserum raised to the N-terminus of *Xenopus* plakoglobin also cross reacts with a 70kDa protein in *Dictyostelium* (XPGN). An antiserum raised to the C-terminus of*Xenopus* plakoglobin does not (XPGC, lane D). XPGC cross-reacts with the positive keratinocyte control (XPGC, lane K): the smaller band may indicate degradation.

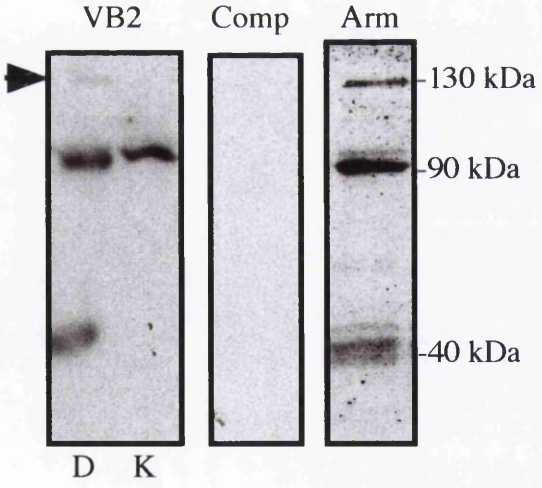

Figure 3.1b *Dictyostelium* first finger/slug whole cell extracts probed with antisera to ß-catenin and Armadillo.
An antiserum raised to the C-terminal peptide of human ß-catenin recognises 90, 40 and 130kDa (arrowhead) proteins in *Dictyostelium* (VB2, lane D) and a 90kDa protein in keratinocytes (VB2, lane K). The VB2 signal in *Dictyostelium* can be competed out by preabsorption of the antiserum with the peptide to which it was raised (Comp). Anti-*Drosophila* Armadillo antiserum (Arm) recognises ~~the same~~ those recognised by with the same molecular weight three proteins as VB2 in *Dictyostelium*.

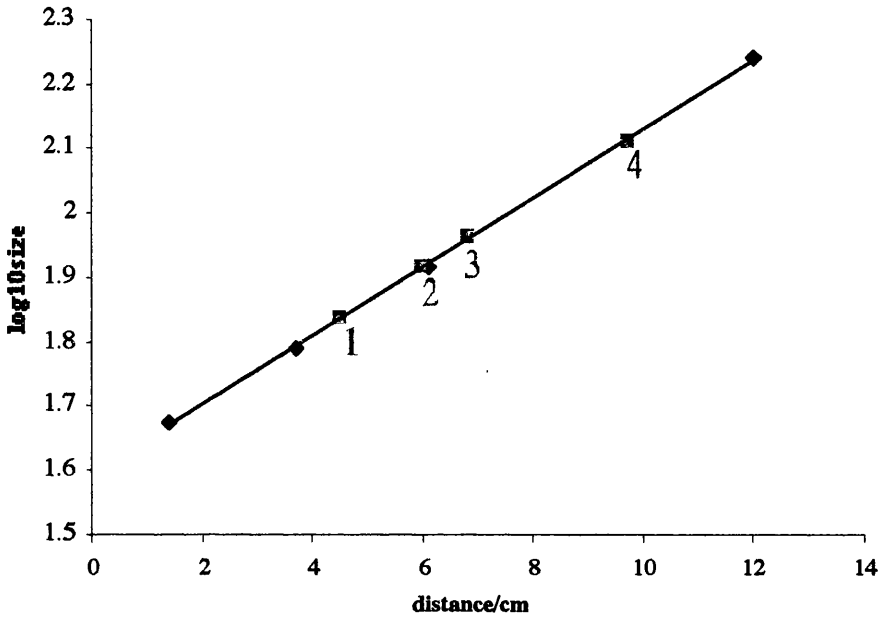

Figure 3.2 Mobilities of Ddplako and Ddarm on SDS-PAGE.

Dictyostelium whole cell extracts were separated by SDS-PAGE on a 7.5% resolving gel, with molecular weight standards (BioRad). The $\log_{10}$ of the size markers was plotted against their distance from the bottom of the gel (black diamonds) and the best fit line calculated by linear regression. The size of Ddplako (1), human plakoglobin (2), Ddarm (3,4) and human ß-catenin (3) was extrapolated from the regression line after measuring their distances from the bottom of the gel (grey squares). The standards used were soybean trypsin inhibitor (47.5kDa), carbonic anhydrase (62kDa), bovine serum albumin (83kDa) and ß-galactosidase (175kDa).

3.3 Expression of both Ddplako and Ddarm is developmentally regulated.

Dictyostelium cells first form cell-cell contacts when they begin to stream, at around 8 hours of development. If Ddplako and Ddarm function in the formation of cell-cell contacts it is likely that their expression will be developmentally regulated. Wild type (AX2) cells were developed on non-nutrient agar. Cells were harvested at different times throughout development and extracts subjected to SDS-PAGE and western blotting with anti-HPGN or a commercial antiserum raised to the same peptide as VB2, which recognises Ddarm. Ddplako is not present in vegetative cells but appears at 8 hours after the onset of starvation (figure 3.3a). Expression remains at the same level throughout development. The presence of a larger band in some lanes is not understood, but is specific to anti-HPGN and is not due to cross-reaction of the secondary antibody. At 8, 16, 18 and 20 hours of development a slightly smaller form of Ddplako is detected, running below the major Ddplako isoform. This could possibly indicate modification or degradation of Ddplako: a similar band has been observed running below plakoglobin in certain cell types (Cowin *et al.*, 1986).

In animal systems Arm/ß-catenin is regulated by gsk3ß during development. To test whether the same regulation occurs in *Dictyostelium*, a developmental time course of *gskA*$^-$ cell extracts was probed with anti-HPGN. Figure 3.3b shows that Ddplako expression is greatly reduced during development in the *gskA*$^-$ mutant. Expression begins around 4 hours later than wild type (compare with figure 3.3a), reaches a peak at around 16 hours and is not maintained later in development (20 hours). The protein loading of all the samples in figure 3.3 is identical, as judged by PonceauS staining (data not shown). It is interesting to note that unlike wild type cells, *gskA*$^-$ cells do not form streams, but form small mounds 10-12 hours after starvation. Thus the expression of Ddplako at 8 hours in wild type cells correlates with the presence of streams. Ddplako could be necessary for streaming to occur: its absence in the *gskA*$^-$ mutant would therefore lead to an inability to stream. Alternatively the expression of Ddplako could be stabilised by the formation of cell-cell contacts in wild type streams.

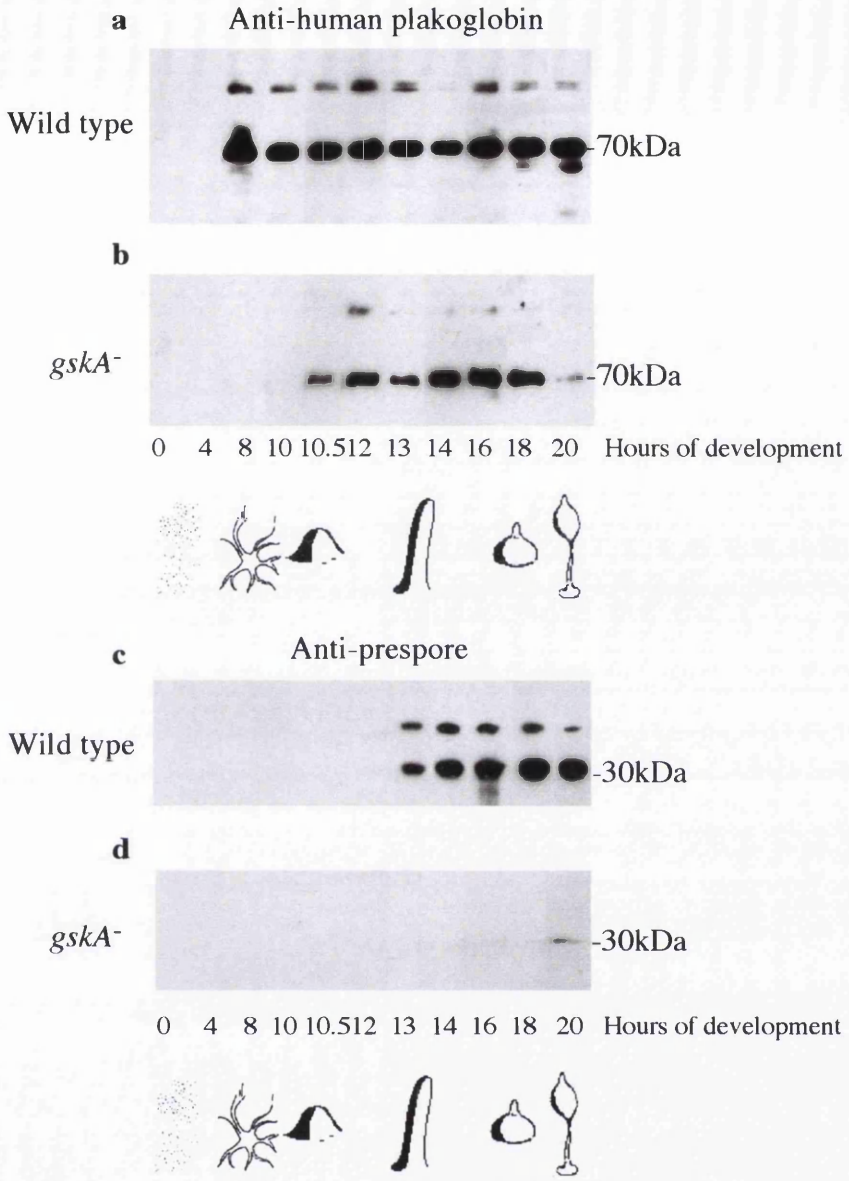

Figure 3.3 Ddplako expression is developmentally regulated.
a) In wild type cells, Ddplako (recognised by anti-HPGN) is absent from vegetative cells and is expressed as cells begin to stream. Ddplako expression persists throughout the rest of development. The identity of the larger cross-reacting band is not known. The slightly smaller band prominent at 20 hours may be a degradation product.

b) Ddplako expression is delayed in *gskA*$^-$ mutant cells until they begin to aggregate into small mounds. Expression levels are lower than in wild type cells and expression does not persist late in development. Note the morphological stages shown apply to wild type: the *gskA*$^-$ mutant does not form streams, although it does form mounds at ~10 hours. In addition *gskA*$^-$ culminants have no discernible sporehead

c) Ddplako does not have the same developmental expression profile as a prespore specific marker in wild type cells. The same blot as in a) was probed with MUD-1 antibody, raised against psA protein.

d) In the *gskA*$^-$ mutant, psA is only detectable at 20 hours of development, unlike Ddplako, which is expressed earlier and to a higher level. The same blot as in b) was probed with MUD-1.

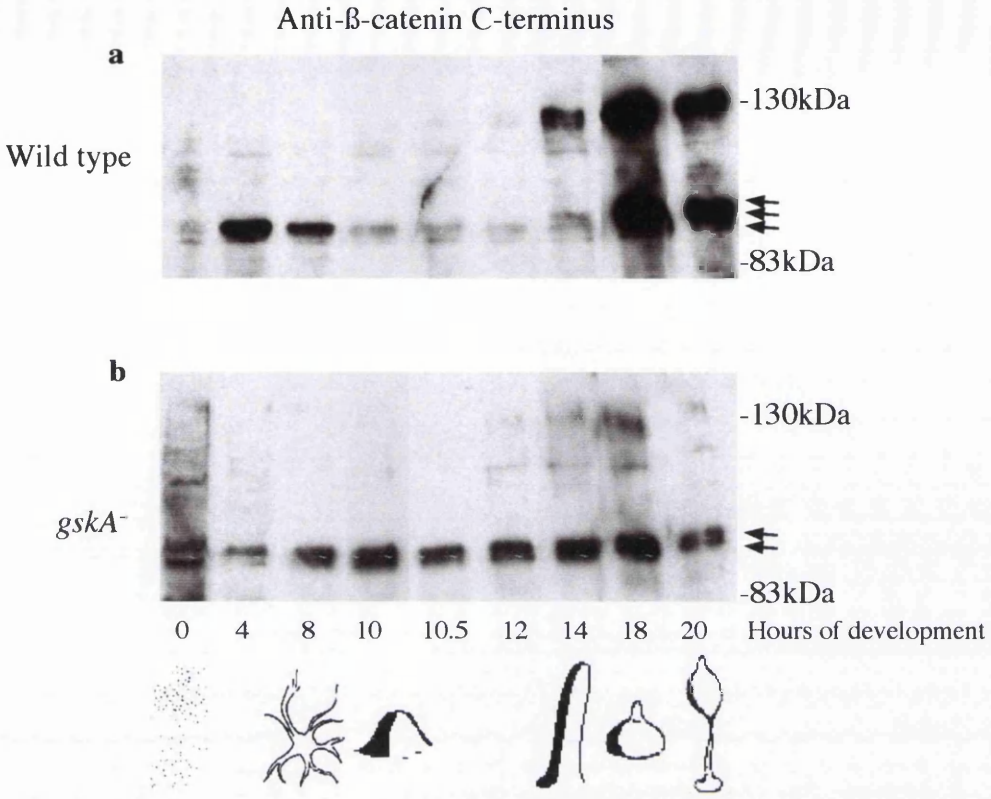

Figure 3.4 Ddarm expression is developmentally regulated.
Developmental western blot time courses were probed with an antiserum to the C-terminal peptide of ß-catenin.
a) 92kDa Ddarm is present in vegetative wild type cells and throughout development. Multiple isoforms are indicated with arrows. Ddarm expression declines between 10 and 12 hours from the levels observed at 4 and 8 hours. A more slowly migrating isoform (top arrow) is detected at 14 hours, which is upregulated during culmination. 130kDa Ddarm is expressed from 14 hours onwards.

b) In the *gskA*⁻ mutant 92kDa Ddarm is not as highly expressed at 4 and 8 hours and is not downregulated between 10 and 12 hours. The slowest migrating form of 92kDa Ddarm is not expressed at all during culmination (arrows) and expression of 130kDa Ddarm is barely detectable compared to the wild type cells.

Another possible explanation for the reduced level of Ddplako in the *gskA*⁻ mutant is that Ddplako is a prespore-specific or prespore-enriched protein. The *gskA*⁻ mutant produces only 5% prespore cells, as opposed to 80% in wild type (Harwood *et al.*, 1995). However, probing the developmental time courses with an antibody, MUD-1, to a prespore-specific marker, psA (Krefft *et al.*, 1983), shows that this is clearly not the case (figure 3.3c,d). In wild type cells Ddplako is first detected much earlier after the onset of starvation (8 hours, figure 3.3a) than psA (13 hours, figure 3.3c). Ddplako is first expressed around 4.5 hours later in the *gskA*⁻ mutant than in wild type cells and its peak expression level almost matches that seen in the wild type. However, psA is barely detectable in *gskA*⁻ cells (figure 3.3d) and is expressed 7 hours later in the *gskA*⁻ mutant (20 hours post-starvation) than in wild type (figure 3.3c,d). Thus the timing and level of expression of psA relative to Ddplako, in both wild type and *gskA*⁻ mutant cells, indicate that Ddplako is not a prespore-specific protein.

The expression pattern of Ddarm in wild type cells is complex (figure 3.4a). The 92kDa protein is present in vegetative cells and throughout development, but its expression is strongest at 4-8 hours and at 18-20 hours, during culmination (figure 3.4a). The lower level of Ddarm seen at 10-12 hours is not due to uneven loading of samples, as judged by Coomassie and PonceauS staining (data not shown). The 130kDa protein is expressed from the slug stage onwards and increases at 18-20 hours. As discussed in section 3.2, the 130kDa protein could be a modified form of the 92kDa protein or could be a closely related protein as the expression and regulation of the two proteins ~~appears to~~ may be linked* (see also figure 3.4b). Interestingly, both the 92kDa and 130kDa proteins appear to run as multiple bands in this experiment (arrows in figure 3.4). At 4-8 hours post-starvation it is the upper band of a 92kDa doublet that is expressed to the highest level (figure 3.4a). At culmination, the 92kDa protein runs as a triplet, as a third, more slowly migrating band is greatly upregulated (figure 3.4a).

The presence of doublets and triplets often indicates a protein modification such as phosphorylation. In animal systems, gsk3ß is thought to phosphorylate and regulate Arm/ß-catenin. In the *Dictyostelium gskA*⁻ mutant, the uppermost band of the 92kDa Ddarm triplet is eliminated (figure 3.4b), never appearing during culmination. Likewise, no increase in the expression of the upper band in the Ddarm doublet is seen at 4-8 hours (figure 3.4b). In addition, the 130kDa Ddarm protein is present at much lower levels in the *gskA*⁻ mutant than in wild type cells.

* The expression patterns are not identical, however this does not preclude differential post-transcriptional regulation.

3.4 Subcellular localisation of Ddplako protein

Whole cell first finger/slug lysates were separated into three fractions. Firstly, a "soluble cytosol" fraction (S100) was obtained. The remaining pellet was then further fractionated in 1% TritonX-100 containing buffer. This resulted in a "detergent-soluble" fraction (Ps), containing nuclear, membrane-associated and some cytoskeletal proteins and an "insoluble pellet" (Pi). Wild type cells have a substantial pool of "cytosolic"/soluble Ddplako but the majority is found in the membrane/nuclear Ps fraction, with only a trace in the cytoskeletal fraction (figure 3.5a). Similar results were obtained with cells from earlier developmental stages (streams and aggregates, data not shown). Identical results were obtained using 1% NP-40 in the buffer (data not shown). These results are in accordance with data from mammalian epithelial cells showing that the majority of ß-catenin and at least 30% of the plakoglobin (assumed largely desmosomal) is detergent-soluble when treated with 0.5% NP40 (Stewart and Nelson, 1997).

When the distribution of Ddplako between these fractions in wild type and *gskA*$^-$ mutant cells was compared, it was clear that Ddplako was specifically depleted from the membrane-associated (Ps) fraction (figure 3.5a). Soluble (S100) and cytoskeletal (Pi) levels were identical in the wild type and mutant strains (figure 3.5a). As discussed in section 3.3, this could indicate a lack of Ddplako-containing membrane-associated structures in the *gskA*$^-$ mutant, and certainly suggests that "cytosolic" Ddplako stability is not affected by gskA activity.While this work was in progress, the presence of intercellular junctions was discovered in the *Dictyostelium* culminant (M. J. Grimson, unpublished data). The junctions consist of dense actin-containing plaques, abutting the membranes of adjoining cells, which encircle the top of the stalk tube during culmination. Figure 3.5b shows that anti-HPGN antiserum localises to these junctions. This provides further evidence that Ddplako may have true ß-catenin/plakoglobin-like functions in *Dictyostelium.* No junctional localisation of control anti-HPGN preabsorbed with its cognate fusion protein was seen (M. J. Grimson, pers commun).

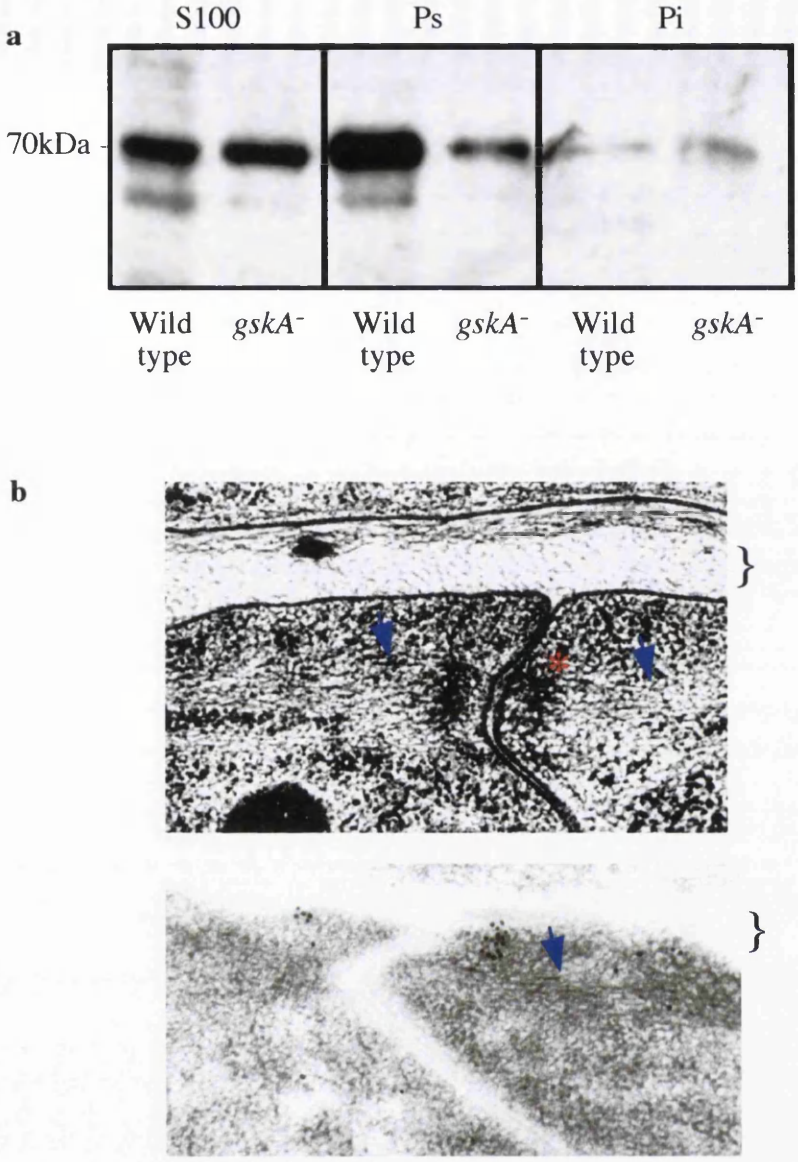

Figure 3.5 Subcellular localisation of Ddplako.

a) Wild type and *gskA*- cells were lysed to release a cytosolic (S100) fraction. The remaining pellet was then further subdivided into detergent-soluble (Ps) and insoluble (Pi) fractions. Fractions were separated by SDS-PAGE and probed with anti-HPGN. The majority of wild type Ddplako was found in the membrane/nucleus-associated Ps fraction. In the *gskA*- mutant Ddplako is specifically depleted from this Ps fraction. The smaller band detected is probably due to degradation of Ddplako during the fractionation procedure.

b) Junctional localisation of Ddplako in the culminant. An intercellular junction, visualised by TEM, is shown (top panel), which consists of a dense plaque (*) with a bundle of actin filamens running away from it (arrows) in cells adjacent to the stalk tube (bracket). The lower panel shows localisation of anti-HPGN visualised with colloidal gold-conjugated secondary antibody (black spots) in the plaque area of two adjoining cells. Again, actin filaments are marked by an arrow, the stalk tube by a bracket.

The photographs in b) were provided by M. J. Grimson

3.5 Discussion

The data presented in this chapter provide evidence for the existence of two distinct Arm/ß-catenin/plakoglobin-like proteins in *Dictyostelium discoideum.* A 69kDa protein (Ddplako) is specifically recognised by antisera raised to the amino-terminus of plakoglobin proteins from two different vertebrate species. An antiserum raised to the C-terminus of plakoglobin does not cross react with this Ddplako. However, Ddplako is slightly smaller than vertebrate plakoglobin and could thus be truncated at the C-terminus relative to its vertebrate counterparts. During development, Ddplako is expressed from the time of aggregation and is found in cytosolic and membrane-associated subcellular pools in biochemical fractionation studies. Importantly, a protein recognised specifically by anti-HPGN, presumably Ddplako, is found in cell-cell junctions during culmination. Levels of Ddplako are reduced in the *gskA*$^-$ mutant and Ddplako expression is delayed relative to wild type. It appears that Ddplako is specifically depleted from a membrane-associated pool in the *gskA*$^-$ mutant. Thus Ddplako expression is altered in the absence of gskA. However, Ddplako does not appear to be destabilised by gskA, and is therefore unlike Arm/ß-catenin, and perhaps plakoglobin, (Bradley *et al.*, 1993), which are upregulated by a Wnt signal and hence lowered gsk3ß activity in metazoa. However, as discussed in chapter 1, Arm/ß-catenin and plakoglobin appear to have different developmental functions and there is no direct evidence that plakoglobin is regulated by gsk3ß homologues *in vivo.* Ddplako could be analogous to vertebrate plakoglobin, having cellular functions independent of gsk3ß activity, for example, in cell-cell junctions. The lowered level of Ddplako in the *gskA*$^-$ mutant could be due to its altered morphology: the *gskA*$^-$ mutant may lack Ddplako-containing junctional structures. Alternatively, Ddplako could be regulated by gskA (directly or indirectly) in a positive manner.

Ddarm runs as 92kDa and 130kDa isoforms on SDS-PAGE. It is specifically recognised by two different antisera raised to the conserved C-terminal peptide of vertebrate ß-catenins and also cross-reacts with an antiserum raised to the C-terminal half of *Drosophila* Arm. The 92kDa Ddarm is present in vegetative cells and throughout development but its levels increase markedly during culmination. The 130kDa Ddarm is expressed from early culmination onwards. Both Ddarm isoforms run as multiple isoforms which are also differentially expressed during development, suggesting that Ddarm may be modified post-translationally, as in other systems. Phosphorylation and ubiquitination of Arm/ß-catenin usually only produce small 7-

10kDa bandshifts on SDS-PAGE (Aberle *et al.*, 1997; Peifer *et al.*, 1994b; Peifer *et al.*, 1994c; Riggleman *et al.*, 1990; Salomon *et al.*, 1997). However, larger forms of ubiquitinated ß-catenin have been observed (Orford *et al.*, 1997). Could differential mRNA splicing generate the 92kDa and 130kDa forms of Ddarm? *Drosophila arm* has two splice variants; ubiquitous *arm* mRNA encodes a protein of 92kDa, while a protein 10kDa smaller is present later in development and is specific to the nervous system (Loureiro and Peifer, 1999). Perhaps 130kDa Ddarm also has a specific function late in *Dictyostelium* development. The expression pattern of 92kDa Ddarm suggests that it could, in part, be regulated by gskA similarly to the regulation of Arm/ß-catenin by gsk3ß in metazoa.

Although gskA protein levels remain constant throughout *Dictyostelium* development, gskA activity increases to peak at around 10 hours in wild type cells (Plyte *et al.*, 1999). This is the time at which lowest levels of the 92kDa form of Ddarm are seen (figure 3.4b), consistent with a model where gsk3ß-mediated phosphorylation of Arm reduces its stability (Peifer *et al.*, 1994b; Peifer *et al.*, 1994c). When gskA activity is low in wild type *Dictyostelium* cells, at pre- and early aggregation and during culmination (Plyte *et al.*, 1999), more Ddarm (92kDa and, during culmination only, 130kDa) is present, again suggesting it is stable in the absence of gskA activity. Also in accordance with the model, 92kDa Ddarm is expressed at a higher level in the *gskA*$^-$ mutant than in wild type, between 10 and 12 hours (figure 3.4b). This is also the case for Arm in *Drosophila zw3*$^-$ mutant embryos (Peifer *et al.*, 1994b; Peifer *et al.*, 1994c). In *zw3*$^-$ mutant embryos the slower migrating, phosphorylated, form of Arm is reduced or eliminated, as seen with Ddarm in *gskA*$^-$ mutants (Peifer *et al.*, 1994b).

However, Ddarm must also be regulated by mechanisms other than that involving gskA. If the slowest migrating forms of Ddarm present in wild type culminants are phosphoproteins, they must either be stabilised in some way or be phosphorylated by kinases that do not target Ddarm for degradation. Perhaps this phosphorylated Ddarm is localised away from the degradation machinery in cell-cell junctions, or is present at levels at which the degradation apparatus becomes saturated. Arm is likely to be phosphorylated by other serine/threonine and tyrosine kinases (Peifer *et al.*, 1994b) and junctional Arm levels are not affected by zw3 activity (Peifer *et al.*, 1994c). Further biochemical experiments, treating cells with specific phosphatases and their inhibitors, or with inhibitors of proteasome-mediated degradation, could shed more light on the regulation of Ddarm. Unfortunately

immunofluorescent staining of *Dictyostelium* cells with anti-plakoglobin or anti-ß-catenin antisera has not yielded any positive results.

In an attempt to clone Ddplako, anti-HPGN was used to screen a *Dictyostelium* first finger/slug λ ZAPII expression library. Similar cross-species screens using monoclonal antisera to bovine plakoglobin have been carried out successfully (Cowin *et al.*, 1986; Franke *et al.*, 1989). However, no positive clones were obtained that could be followed through more than one round of screening. The signal was lost, or the antiserum bound non-specifically to all bacteriophage clones, in secondary and tertiary screens. The library was constructed using oligo-dT primers and thus is likely to have a strong bias towards expressing the C-terminal regions of proteins. A large (~2.5kb) λ clone would be needed to provide expression of N-terminal Ddplako for recognition by anti-HPGN.

In conclusion, I have shown that potential homologues of Arm/ß-catenin and plakoglobin are present in *Dictyostelium* and suggest, for the first time, that both gsk3ß-mediated signalling through these proteins during development and their role in regulated cell-cell adhesion may be conserved in a non-metazoan organism. However, only the cloning of genes for Ddarm and Ddplako will prove whether they are, indeed, genuine homologues of their metazoan counterparts, and will provide further insights into their functions and locations during development.

Chapter 4

Cloning of *aardvark*, a *Dictyostelium armadillo*-related gene

4.1 Introduction

Having ascertained that Armadillo-like proteins are present in *Dictyostelium*, the next step was to clone their cognate genes. As mentioned in chapter 3, the first approach taken was an unsuccessful expression library screen with an anti-plakoglobin antiserum. A DNA-based approach was then attempted: short degenerate oligonucleotides were designed based on the sequences of highly conserved regions of metazoan ß-catenin/plakoglobin proteins. These oligonucleotides were used to PCR amplify *armadillo*-related genes and to screen a *Dictyostelium* cDNA library (see section 4.10). Neither approach yielded *armadillo*-related clones.

The Japanese *Dictyostelium* cDNA project (Morio *et al.*, 1998)[1] is a database similar to expressed sequence tag (EST) databases from other organisms. The database contains partially sequenced developmentally regulated cDNA clones isolated from slug stage cells, which can be searched using a BLAST server (Altschul *et al.*, 1997)[2]. This project yielded a cDNA clone encoding a putative protein with homology to Arm/ß-catenin/plakoglobin. This sequence information allowed the subsequent cloning of a full-length cDNA for a developmentally regulated gene, which was named *aardvark*.

4.2 Cloning and sequencing of multiple independent *aardvark* cDNAs

Translated DNA sequence from the 3' end (606bp) of cDNA clone SSF518 (Morio *et al.*, 1998) was found to have similarity (E=0.001) to Arm/ß-catenin and plakoglobin, when a cross-species protein sequence database was searched using BLAST (Altschul *et al.*, 1997). The cloned cDNA, a gift from Dr. Y. Tanaka, comprises a *Dictyostelium* cDNA ligated directionally into the *Sal*I (5') and *Not*I (3') sites of pBluescript II KS- (Stratagene) (Morio *et al.*, 1998). Initial sequencing runs were carried out with standard T3 and T7 primers which anneal within the vector sequence (Stratagene) and one primer, SSF518b, was designed to continue sequencing upstream from part of the known sequence (figure 4.1). Sequence data was assembled into contigs using DNAstar™ software and additional primers were designed in order to sequence over the whole SSF518 cDNA (figure 4.1, dark blue arrows).

In order to obtain independent cDNA clones, a λZAPII slug cDNA library was probed with a ~460bp internal PCR fragment amplified using primers SSF518b and

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[1] Supported by the Japan Society for the Promotion of Science (RFTF96L00105) and the Ministry of Education, Science, Sports and Culture of Japan (08283107).
http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html
[2] http://www.dna.affrc.go.jp/htdocs/Blast/blast2.html

SSF518c (figure 4.1). Eight independent positive clones were obtained after three rounds of screening of which five (all different sizes) were chosen for sequencing. The strategy used to obtain cDNA fully sequenced on both strands is outlined in figure 4.1. The longest clone, clone 1, consists of a fusion with an unrelated clone (identical to SSL547 from the Japanese sequencing project) at the 5' end (figure 4.1). The full length consensus cDNA sequence presented in this chapter was assembled using only the region of clone 1 which was identical to the sequences obtained from other clones (0 kb onwards in figure 4.1). The restriction sites predicted to be present in the sequence (using DNAstar™ software) were confirmed by digesting DNA from each clone with the appropriate restriction enzymes (figure 4.1).

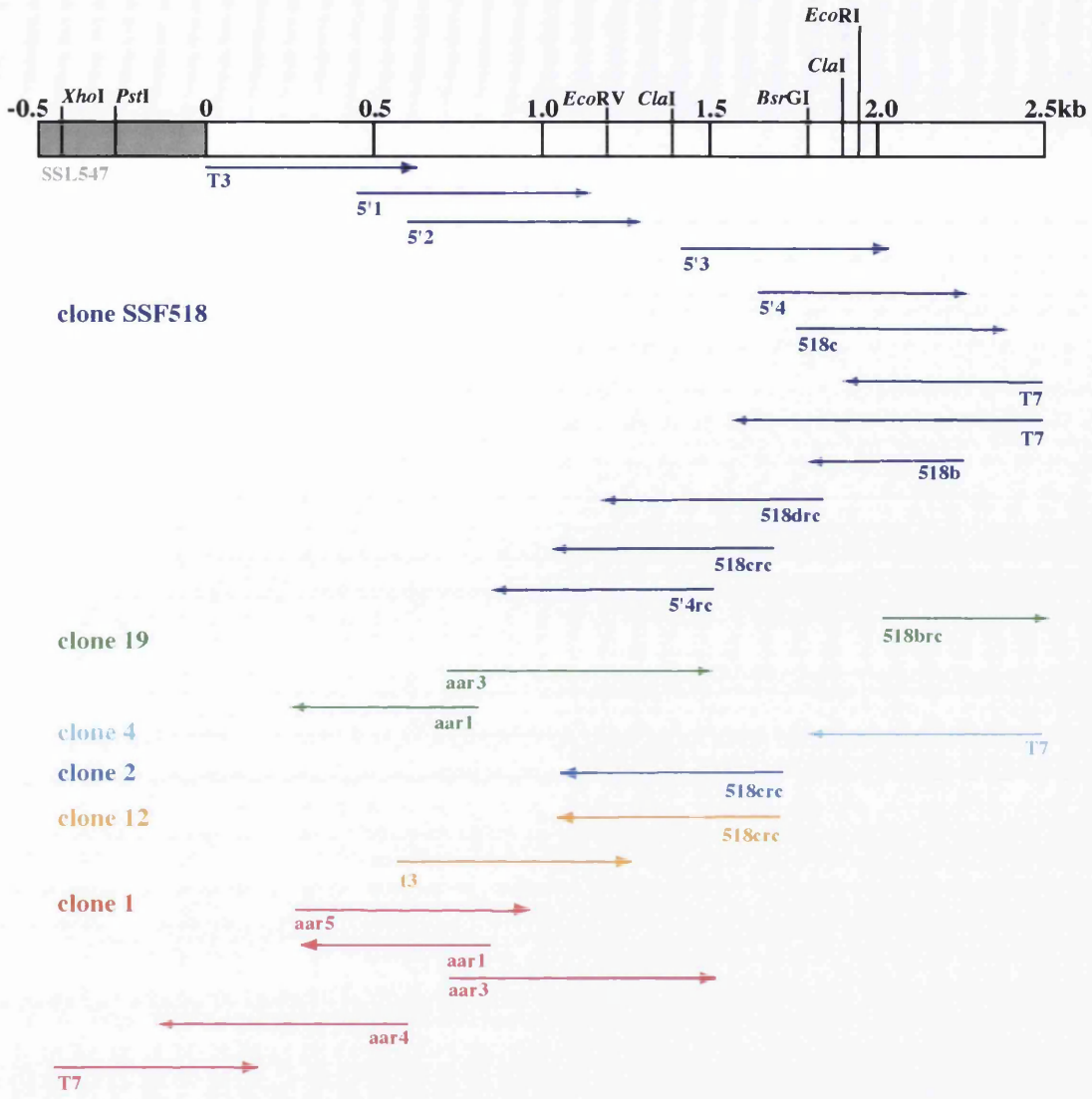


Figure 4.1 Restriction map and sequencing strategy for *aar* cDNAs.
6 independent clones of various sizes were isolated and primers designed such that both strands were sequenced in full to obtain a consensus. The orientation of the clones in pBluescript varied.
Clone 1 (3kb) is a fusion of *aar* (2.5kb) and another unrelated cDNA (SSL547) at the 5' end, shown in grey.

4.3 Full length *aardvark* sequence

The consensus 2473bp cDNA sequence is shown in figure 4.2. The cDNA was named *aardvark* due to its homology to *armadillo* (section 4.3.1). The longest translatable open reading frame (697 amino acids, figure 4.2) is immediately preceded by a long run of deoxyadenosine (A) residues; this is characteristic of the 5' untranslated region *Dictyostelium* genes. Thus although a classical Kozak translation initiation consensus sequence (Kozak, 1986) is not present upstream of the initiating ATG codon, it is probable that a full-length protein sequence has been obtained.

4.3.1 Aardvark protein contains Armadillo repeats

The C-terminal two thirds of the translated Aar protein consists of ten Armadillo (Arm) repeats, indicated by the blue and yellow boxes in figure 4.2. The repeat boundaries marked were those defined by the X-ray crystal structure of ß-catenin (Huber *et al.*, 1997a), which are shifted by half a repeat compared to the original consensus repeat sequence (Peifer *et al.*, 1994a). Analysis of the sequence by eye suggested that when compared to vertebrate Arm repeat proteins, the Aar repeats are more similar to the consensus for plakoglobin, ß-catenin and Arm than to other family members such as p120 or importin-α (Peifer *et al.*, 1994a). This was confirmed by performing BLAST searches (Altschul *et al.*, 1997) with the full-length Aar protein sequence or the repeat region alone. Aar sequence has higher homology to *Xenopus* and human plakoglobin and ß-catenin/Armadillo (probabilities from e^{-9} to e^{-6}), than to importin-α (e^{-4}), p120 proteins or smgGDS/Darlin (~0.01).

The ten Aar repeats were aligned with each other by eye and compared to the consensus Arm/ß-catenin/plakoglobin repeat (figure 4.3, (Peifer *et al.*, 1994a)). The residues which match the Aar repeat majority are marked in green; structurally conserved changes (e.g. hydrophobic, basic or acidic substitutions, which are known to exist within other Arm repeats (Huber *et al.*, 1997a), are marked in blue.

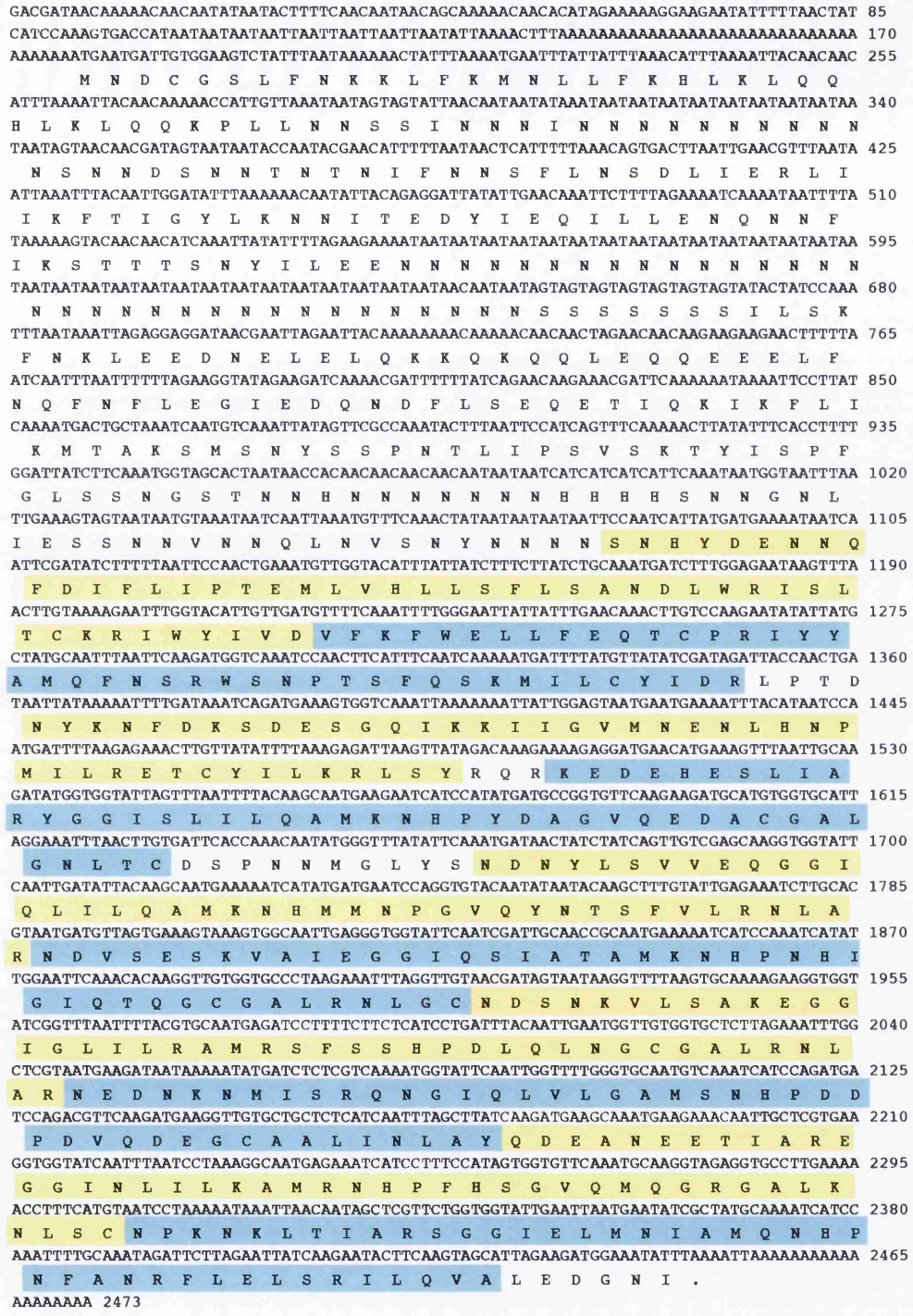


Figure 4.2 ***Aardvark*** **cDNA sequence.**
Nucleotide positions are numbered on the right. The inferred amino acid sequence is shown below the nucleotide sequence using the single letter amino acid code. The ten Armadillo repeats are highlighted by yellow (odd repeats) and blue (even repeats) boxes.

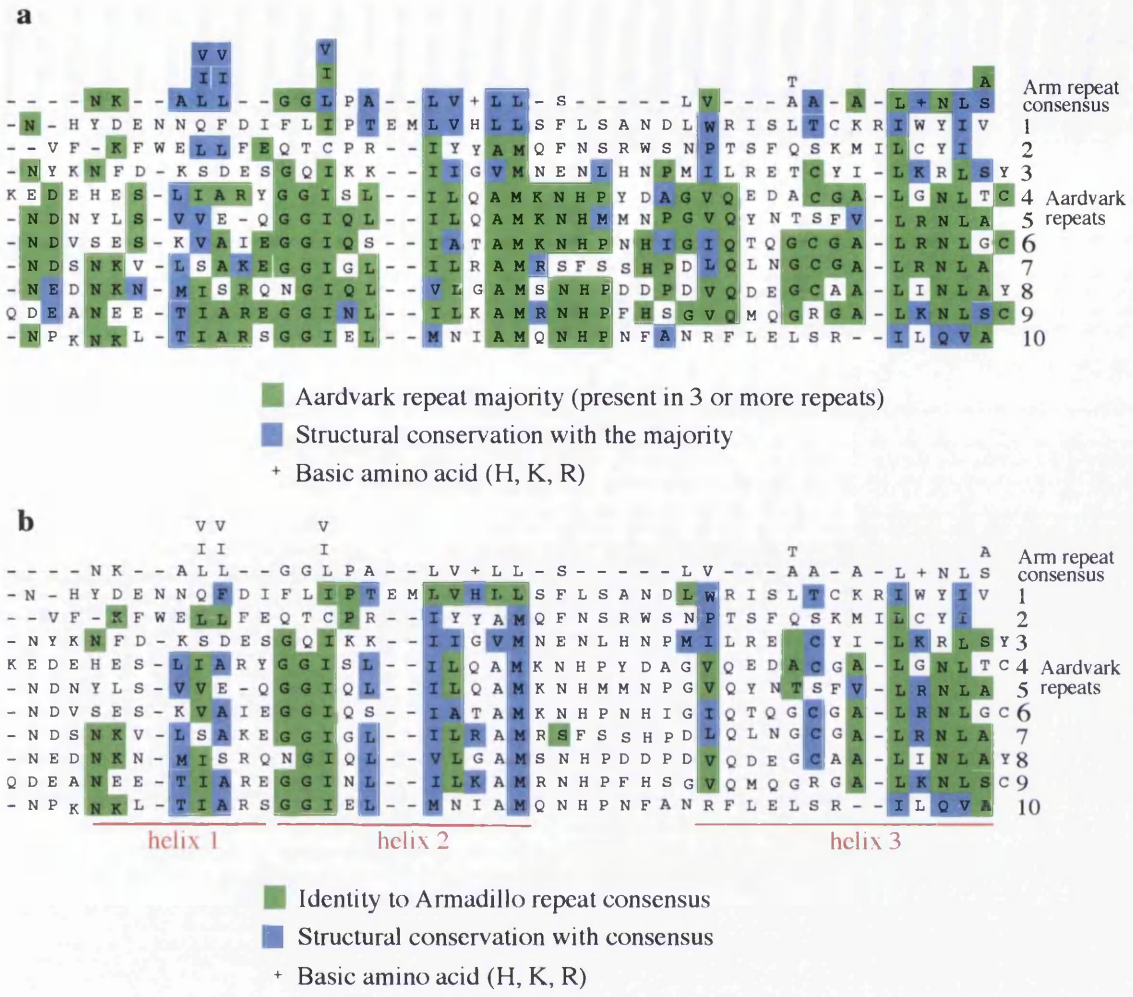


Figure 4.3 Armadillo repeats of Aardvark.

a) Comparison of Aar repeats with each other. The residues which constitute the Aar majority are highlighted in green. Residues showing structural conservation with the majority are highlighted in blue. The Arm repeat consensus sequence is derived in part from Peifer *et al* (1994a) with slight modifications defined by Huber et al (1997). Conservative changes are defined as those allowed within the three-dimensional structure of the ß-catenin repeat region (Huber *et al*, 1997a).

b) Comparison of Aar repeats with the Arm consensus. Residues identical to the consensus are green, structurally conserved residues are blue, as in a). The areas with greatest conservation are those which form the three α-helices within the repeats (Huber *et al*, 1997a; marked in red). Aardvark repeat 1 is quite different in sequence to the other nine repeats and shows greater identity to the consensus in the helix 2 region.

4.3.2 How does Aardvark differ from ß-catenin/plakoglobin?

A problem with the analysis of Aar arises from the repetitive nature of the protein sequence. No direct comparison can be made between the whole ten-repeat region of Aar and proteins such as ß-catenin and plakoglobin, which contain twelve repeats. In contrast, a *Dictyostelium* smgGDS (Darlin) has been cloned which has eleven Armadillo repeats, as do its mammalian counterparts (Vithalani *et al.*, 1998). BLAST searching defines Aardvark as being more similar to *S. cerevisiae* Vac8p (Fleckenstein *et al.*, 1998; Pan and Goldfarb, 1998; Wang *et al.*, 1998) and related *A. thaliana* and human ESTs ($E=e^{-15}$) than to either plakoglobin or ß-catenin. However, close inspection of the BLAST alignments shows that this high probability was generated in part by aligning multiple fragments of Aar sequence against a single region of Vac8p sequence. This highlights a pitfall of trying to align proteins containing degenerate repeats by this method. However, Vac8p does appear to have a catenin-like function in a unicellular organism, as it links a membrane to the actin cytoskeleton; the function of the plant and human genes is unknown. It is interesting to speculate that Aar could have an uncharacterised human homologue and thus define a novel cross-species Arm protein subfamily. Figure 4.4a shows that when a phylogenetic tree is constructed to compare the full length protein sequence of Aar with its relatives from mammals, yeast, plants and *Dictyostelium*, Aar seems to be as divergent from ß-catenin/plakoglobin as it is from any other Arm family protein. The termini of such proteins tend to be more divergent than the repeat regions; indeed Aar has a very short C-terminus (6 amino acids, figure 4.2) compared to its metazoan counterparts (~50-100 amino acids, e.g. (Peifer and Wieschaus, 1990)). Thus a phylogenetic tree comparing the repeat regions of Aar and its relatives was constructed, which also defines Aar as a divergent protein (figure 4.4b). Figure 4.3 shows that Aar contains Arm repeats more similar to one another than to the consensus. This large evolutionary drift between Aar and other proteins could be due in part to the AT-rich codon bias present in *Dictyostelium.*

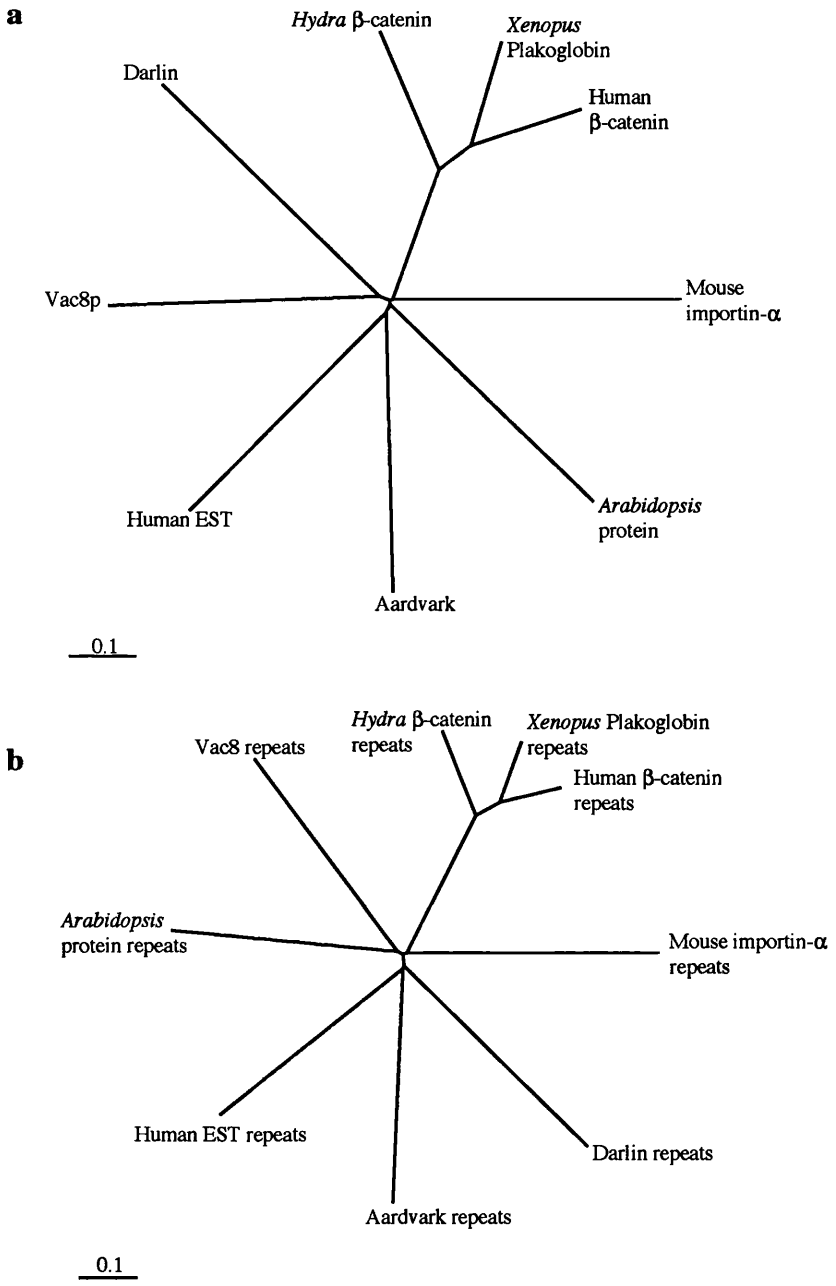


Figure 4.4 Alignments of Aardvark and related proteins.
Aardvark and the proteins most similar to it in a BLAST search were aligned using CLUSTAL W and displayed as a phylogenetic tree using Tree View software (Page, 1996). The lengths of the branches are proportional to the number of amino acid changes between proteins (scale bar indicates 1 change per 10 residues).
a) Phylogenetic tree of full length proteins.
b) Phylogenetic tree of the Armadillo repeat domains.
Database accession numbers: *Arabidopsis* protein, gi/2344894; Darlin, gi/3523097; Human EST, gi/4106984; Human ß-catenin, gi/4503131; *Hydra* ß-catenin, gi/2133442; Mouse importin-α, sp/P52293; vac8p, gi/1077594; *Xenopus* plakoglobin, sp/P30998.

4.3.3 Aardvark has potential sites for gskA phosphorylation and α-catenin binding

In addition to analysing the repeat region of Aar, the Aar sequence was examined for domains that could indicate a conserved function (figure 4.5). The amino-terminus of Aar is rich in runs of asparigine residues, which are common in *Dictyostelium* proteins, and bears no significant similarity to other proteins in the database when analysed by BLAST. However, close inspection of the amino terminus reveals twelve potential sites for gskA/gsk3ß-mediated phosphorylation (S/TxxxS/T, where x is any amino acid: red boxes in figure 4.5). Not all of these are necessarily *bona fide* sites, but seven are flanked by proline or acidic residues as is common with other *in vivo* gsk3ß sites ((Welsh *et al.*, 1996), figure 4.5). Four of the sites are in tandem: ß-catenin/Arm and plakoglobin have three tandem gsk3ß sites in their amino terminus (Peifer *et al.*, 1994a). However, unlike Arm, none of the Aar gskA sites coincide with a consensus ubiquitination motif, DSGxxS, for targeting to proteasomal degradation (Aberle *et al.*, 1997; Orford *et al.*, 1997). Aardvark does not contain any predicted sites for acylation similar to those in Vac8p (Pan and Goldfarb, 1998; Wang *et al.*, 1998).

Could Aar bind a *Dictyostelium* α-catenin homologue? The α-catenin binding site in mammalian ß-catenin and plakoglobin has been mapped to 29 amino acids N-terminal to the first Arm repeat and may form an α-helix with a hydrophobic face (Aberle *et al.*, 1996; Huber *et al.*, 1997b). The first repeat of Aar contains a sequence that bears some resemblance to an α-catenin binding site: three residues are identical, a further seven are structurally conserved (hydrophobic or charged substitutions, figure 4.5). Residue 25 is a phenylalanine in Aar and this aligns with a tyrosine in ß-catenin/plakoglobin. This Y-F substitution is known to retain α-catenin binding function in the mammalian proteins (Aberle *et al.*, 1996). However, a number of residues known to be essential for binding activity in ß-catenin/plakoglobin (red amino acids in figure 4.5) do not appear to be conserved in Aar. In particular, the D-A substitution at position 28 is known to abolish α-catenin binding function *in vitro* (Aberle *et al.*, 1996). It is of note that the first Arm repeat of Aar is quite different to the other nine repeats and the central portion has stronger identity to the Arm repeat consensus (IPxxxLVHLL) than any of the other repeats (figure 4.3). This could indicate evolutionary pressure for conservation of functional α-catenin binding residues within repeat 1. The crystal structure of ß-catenin suggests that its first Arm repeat is a

"half-repeat" and the N-terminal portion of this repeat is disordered. The α-catenin binding site (Aberle *et al.*, 1996) lies where the first helix would be expected, based on the crystal structure (Huber *et al.*, 1997a). This could also be true for Aar as the first half of repeat 1 does not fit the Arm consensus particularly well, compared to the other nine repeats (figure 4.3).

To test whether the putative α-catenin binding site in Aar functions *in vivo* would require cloning or purification of a *Dictyostelium* α-catenin homologue. It is therefore of interest that a cDNA with homology to α-catenin and its relative, vinculin (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991) exists in the cDNA sequencing project database (Morio *et al.*, 1998), clone SSK589).

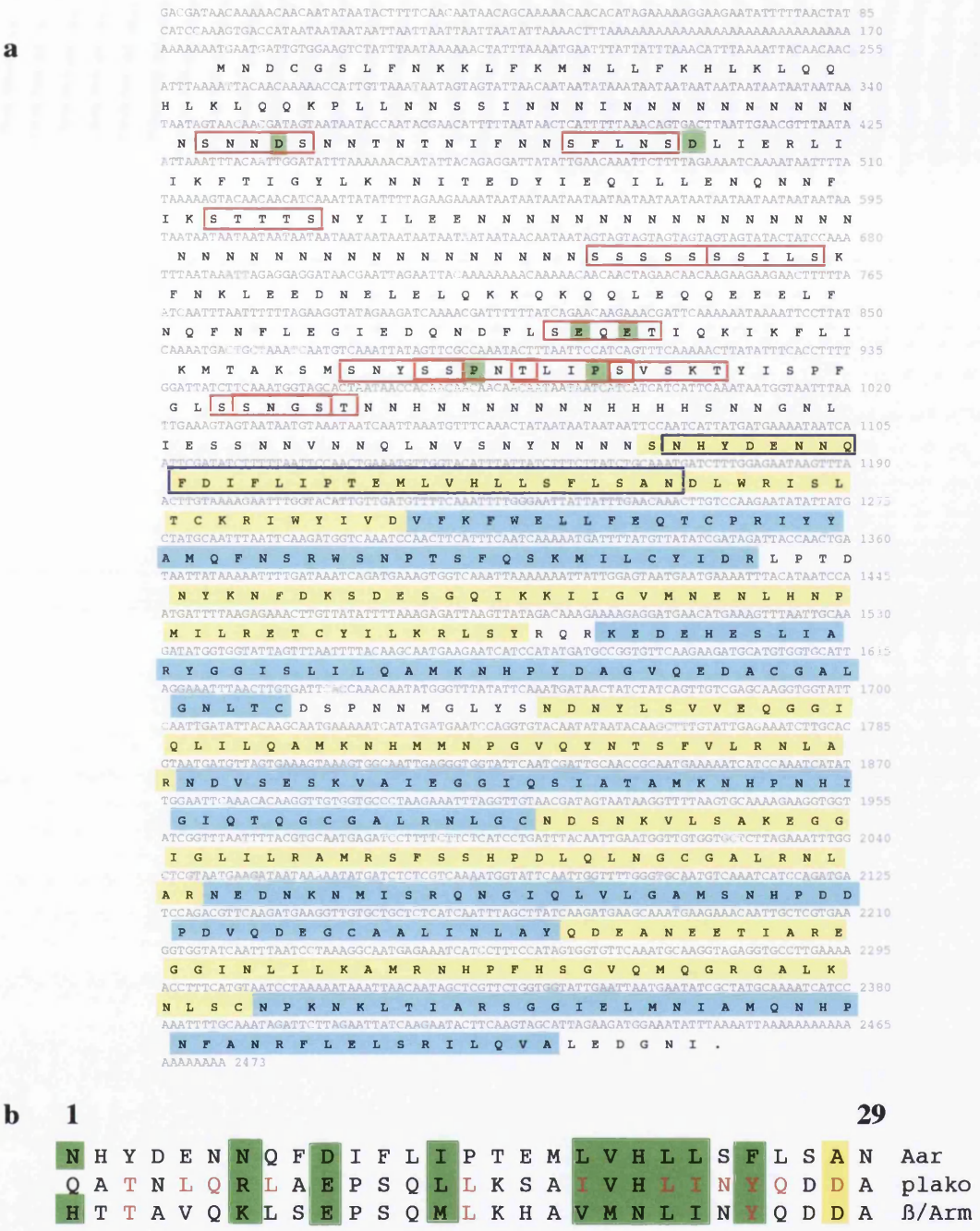


Figure 4.5 Potential gsk3 phosphorylation sites and α-catenin binding region in Aardvark.

a) The putative gsk3 phosphorylation sites are outlined in red. Nearby acidic (D,E) and proline (P) residues are highlighted in green. The potential α-catenin binding region (29 amino acids in Armadillo repeat 1) is outlined in dark blue.

b) Comparison of the potential α-catenin binding region in Aar (top line) with that of plakoglobin (middle) and ß-catenin/Armadillo (bottom). Residues which, when mutated to alanine, abolish α-catenin binding to plakoglobin/ß-catenin/Arm *in vitro* are shown in red (Aberle et al., 1996). Residues in plakoglobin/ß-catenin which are identical or structurally conserved in Aar (hydrophobic, charged or Y-F substitutions) are highlighted in green. The aspartate at position 28 in plakoglobin which abolishes binding to α-catenin when mutated to an alanine (Aberle et al., 1996) is highlighted in yellow.

4.4 Genomic organisation of the *aardvark* locus

4.4.1 *aar* has no close homologues in *Dictyostelium*

Dictyostelium wild type (AX2) genomic DNA was digested with various restriction enzymes and probed at high stringency with the 460bp *aar* PCR product used to screen the λ library. The resulting Southern blot and deduced restriction map is shown in figure 4.6. As predicted from the cDNA sequence and digests, the *aar* gene contains one *Eco*RV site, two *Cla*I sites and a *Bsr*GI site. When probed at lower stringency (using the protocol for low stringency library screening), no additional hybridising DNA fragments were seen, suggesting that no genes closely related to *aar* at the nucleotide level are present in *Dictyostelium* (data not shown). This shows that *aar* is unique and is not part of a gene family caused by gene duplication.

4.4.2 Generation of an *aar*⁻ knock-out mutant

In order to generate an *aar*⁻ mutant by homologous recombination, a construct was designed in which a 600bp *Cla*I-*Eco*RI fragment, from the repeat region of the SSF518 cDNA in pBluescriptII, was replaced with a 1.4kb *Cla*I-*Eco*RI fragment containing the blasticidin-resistance cassette (figure 4.7). Thus homologous genomic recombinants will be a) blasticidin resistant and b) unable to express full-length *aar* mRNA or resulting protein. The vector DNA was removed from the construct after digestion with *Sal*I and *Not*I and the *aar*-blasticidin fragment transformed into *Dictyostelium* AX2 cells by electroporation. Blasticidin-resistant cells were grown to confluence in liquid culture and plated onto SM/*Klebsiella*. Cells from a range of independent colonies with different phenotypes were grown up for preparation of genomic DNA. Several independent knock-out strains were identified by Southern blotting, using full-length *aar* cDNA as the probe (figure 4.7). Insertion of the blasticidin resistance cassette into the *aar* gene leads to loss of one *Cla*I site, abolishing the wild type 500bp *Cla*I fragment and creating a new 2.8kb fragment. In addition, and an 800bp increase in the size of the 1.5kb *Eco*RV fragment is brought about as expected (figure 4.7). The *Bsr*GI site present in the cDNA is lost, and another *Bsr*GI site introduced, due to a *Bsr*GI site present at the extreme 5' end of the blasticidin resistance cassette fragment. Thus an increase in size of the 8kb band is seen (to ~10kb), and a faint ~2.3kb *Bsr*GI band is also present in each knock-out (figure 4.7). If the *aar* gene contains an intron it is not in the region between nucleotides 671-2022, as amplification

of this region by PCR from genomic DNA or cDNA produces identical sized bands of around 1.4kb (figure 4.7)

4.5 *aar* is expressed late in development.

Total RNA was prepared from wild type (AX2) and *aar*$^-$ cells and analysed by northern blotting with a full-length *aar* cDNA probe (figure 4.8). *aar* mRNA is approximately 2.3kb in size, correlating well with the size of the longest isolated cDNA clone. *aar* mRNA is detected from 15 hours of development (first finger/slug stage) onwards in wild type cells and is completely absent from the *aar*$^-$ mutant cells. *aar* expression increases throughout late development, with the highest level of expression at culmination (20 hours, figure 4.8). This suggests a role for *aar* in late development and culmination, although *aar* could be expressed at low levels earlier in the developmental cycle. The presence of *aar* expression at earlier developmental stages might be detected after enrichment of the message by preparation and blotting of polyA$^+$ mRNA.

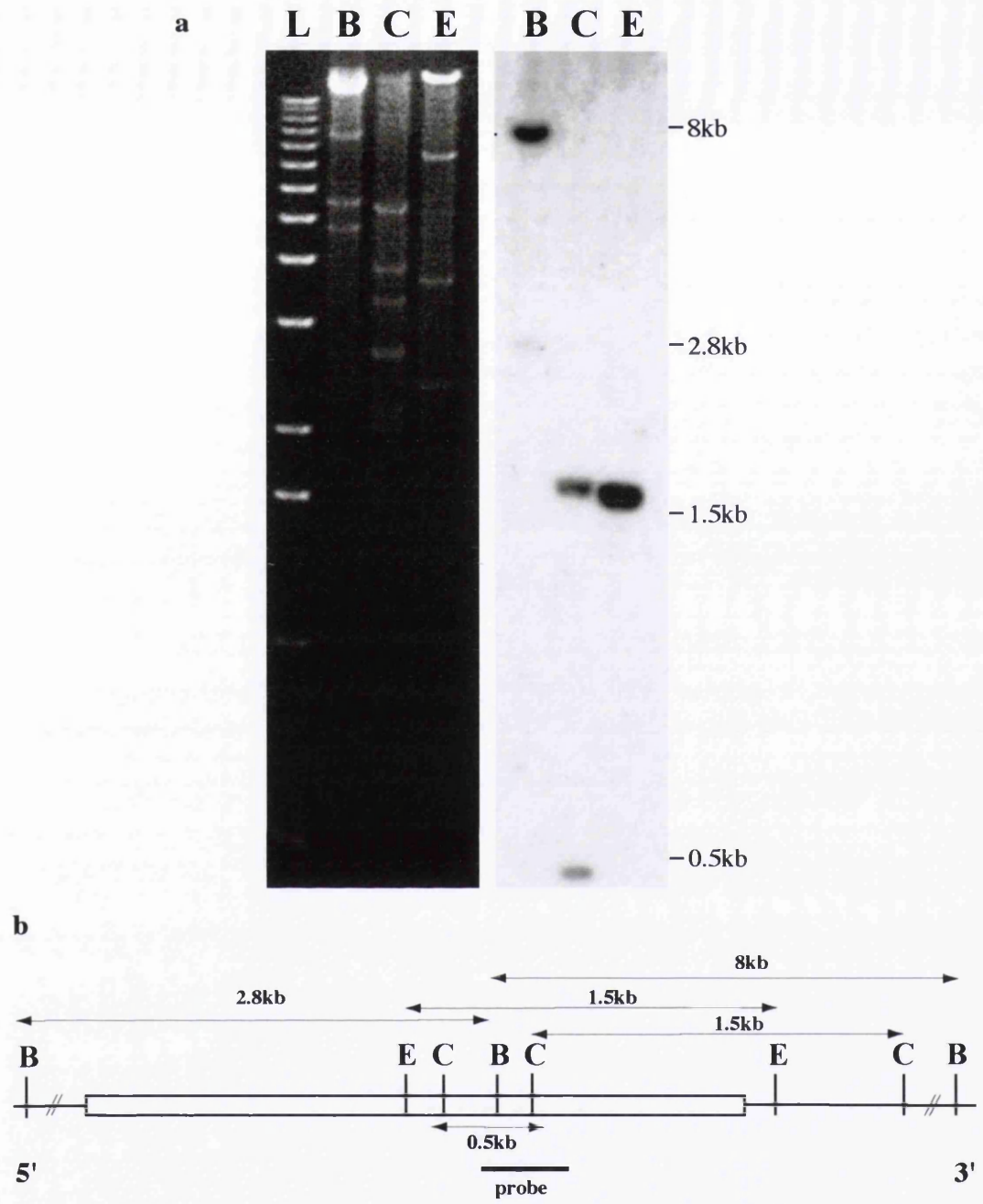


Figure 4.6 The *aar* genomic locus

a) Wild type (AX2) genomic DNA was digested with *Bsr*GI (B), *Cla*I (C) and *Eco*RV (E) and probed with a small PCR fragment amplified from *aar* cDNA. The genomic DNA gel is shown on the left, the Southern blot on the right. L= λ 1kb DNA ladder. Sizes of the Southern blot fragments are shown on the right.

b) The deduced map of the *aar* gene (rectangle) and surrounding DNA. The region homologous to the probe is indicated.

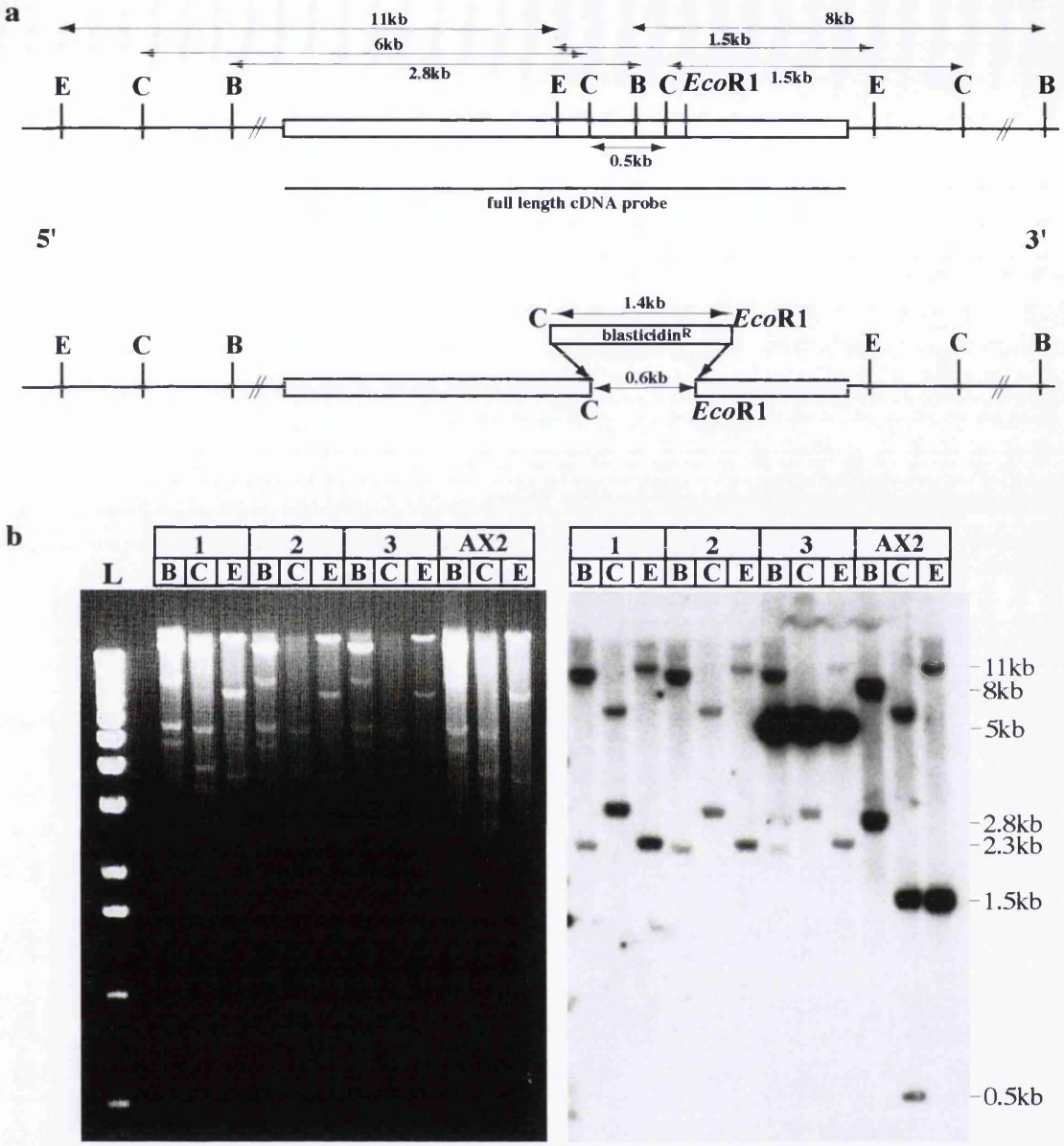


Figure 4.7 Generation of *aar*⁻ mutants.

a) Restriction maps of wild type (top) and *aar*⁻ (bottom) genomic DNA showing insertion of the blasticidin resistance cassette in the knockouts.

b) Genomic DNA from wild type (AX2) and three independent *aar*⁻ mutant clones (1, 2, 3) was digested with *Bsr*GI (B), *Cla*I(C) and *Eco*RV (E) and probed with full length *aar* cDNA. The agarose gel is shown on the left, the Southern blot on the right. The strong 5kb band in clone 3 is likely to be plasmid contamination.

c) PCR amplification of nucleotides 671-2025 from wild type genomic DNA (gDNA) or *aar* cDNA yields identically sized products, suggesting there is no intron in this region of the *aar* gene.

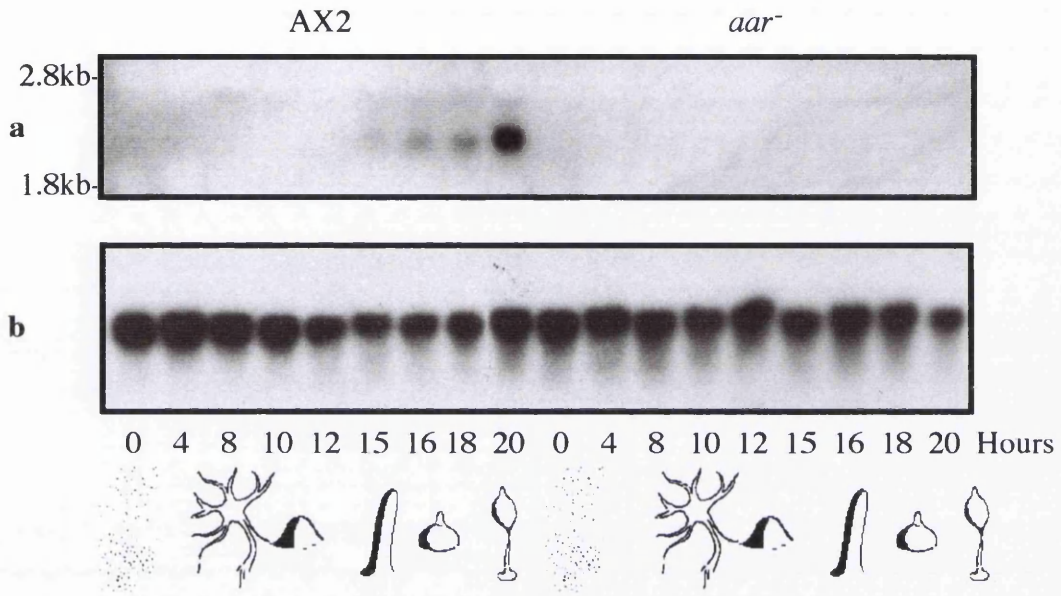


Figure 4.8 *aar* is expressed late in development.
Total mRNA from various developmental stages (0-20 hours, as shown below the blots) was probed with a full length *aar* cDNA probe.
a) In wild type (AX2) cells *aar* message is first detected at 15 hours and increases to a maximal level by 20 hours. No message is seen at any stage of development in the *aar*⁻ mutant.
b) The same blot was reprobed with *Ig7* plasmid DNA (provided by E. Dalton) as a loading control.

4.6 Morphology of *aar*⁻ mutants

4.6.1 *aar*⁻ cells grow normally

Dictyostelium proteins with cytoskeletal functions often have defects in growth and cytokinesis, however this is not the case for *aar*⁻. Wild type (AX2) and *aar*⁻ cells were seeded at low density (1-2 x 10^5 cells/ml) in shaking axenic culture and allowed to grow for several days until stationary. Cells were counted every day. The experiment was repeated three times, each time using a fresh inoculum of wild type and *aar*⁻ cells. Figure 4.9a shows that the growth characteristics of wild type and *aar*⁻ cells are identical, as would be expected from the expression pattern of *aar* mRNA.

4.6.2 *aar*⁻ fruiting bodies have disrupted morphology

Observation of *aar*⁻ mutants by light microscopy suggested that the culminants collapsed onto the substratum at high frequency. This was most clearly seen by scanning electron microscopy (SEM). SEM of rapidly frozen preculminants developed on nitrocellulose filters showed that the morphology of *aar*⁻ during culmination is perturbed. Culmination involves a complex series of cell movements (Dormann *et al.*, 1996; Jermyn *et al.*, 1996; Jermyn and Williams, 1991; Sternfeld, 1992; Sternfeld and David, 1982). When wild type slugs begin to culminate, prestalk cells move towards the tip and enter the top of the developing stalk tube. As the stalk elongates the sporehead is lifted off the substratum. *aar*⁻ cells begin culmination normally (figure 4.9b). However, as culminants form, their stalks appear thin and weak (figure 4.9c,d,e) and collapse, contacting the substratum. Multiple attempts to raise the sporehead occur (figure 4.9d). The slime sheath around the stalk and spore mass is often disrupted (4.9d,e and figure 4.10). Eventually most fruiting bodies fall over and lie on the substratum and the sporehead may break open (figure 4.10). SEM of late culminants showed that a new attempt at development occurred from within these terminal structures (figure 4.10). I termed this process "reculmination". Reculminants range from small bumps (4.10a,b), to tipped mounds (4.10c) to structures resembling an aerial sporehead (4.10d). Wild type culminants do not form reculminants, even when they have collapsed onto the substratum (figure 4.10e).

Prestalk cells from the tip of the slug form the tip of the culminant, prior to their entry into the stalk tube (figure 4.9f). The morphology of the tip of *aar*⁻ fruiting bodies is aberrant: fruiting bodies generally have enlarged tips, rather like the tip of a slug

(figure 4.9g, compare with wild type, figure 4.9f). Some fruiting bodies have extra protrusions, resembling tips, emanating from the developing spore mass (figure 4.9h).

4.6.3 *aar*⁻ mutants have no intercellular junctions around the stalk tube

The structure of the *aar*⁻ mutant culminants was examined in detail. As described in chapter 3, *Dictyostelium* has intercellular junctions, which organise a ring of actin filaments. The actin ring is present in a collar of cells that surround and constrict the top of the stalk tube (M. J. Grimson, unpublished data, and figure 4.11a,b,c). Light microscopy of longitudinal sections from *aar*⁻ preculminants shows that *aar*⁻ mutants do not have a constriction at the top of the stalk tube (figure 4.11d, compare with 4.11a). Cross sections of *aar*⁻ culminants completely lack identifiable junctions or an actin ring, when examined at high magnification by transmission electron microscopy (TEM, figure 4.11e). The cells that normally contain junctions lose their organised arrangement and polarity. The double layer of cellulose, which normally surrounds the vacuolating stalk cells, is not present in this area in the *aar*⁻ mutant. Where cellulose is seen, it is arranged in highly disorganised patches (figure 4.11e). Hence Aar appears necessary for the formation of junctions, which organise actin filaments to constrict the top of the stalk tube. The presence of intercellular junctions and the actin ring appears to be instrumental for initiating the correct deposition of cellulose at the top of the developing stalk tube.

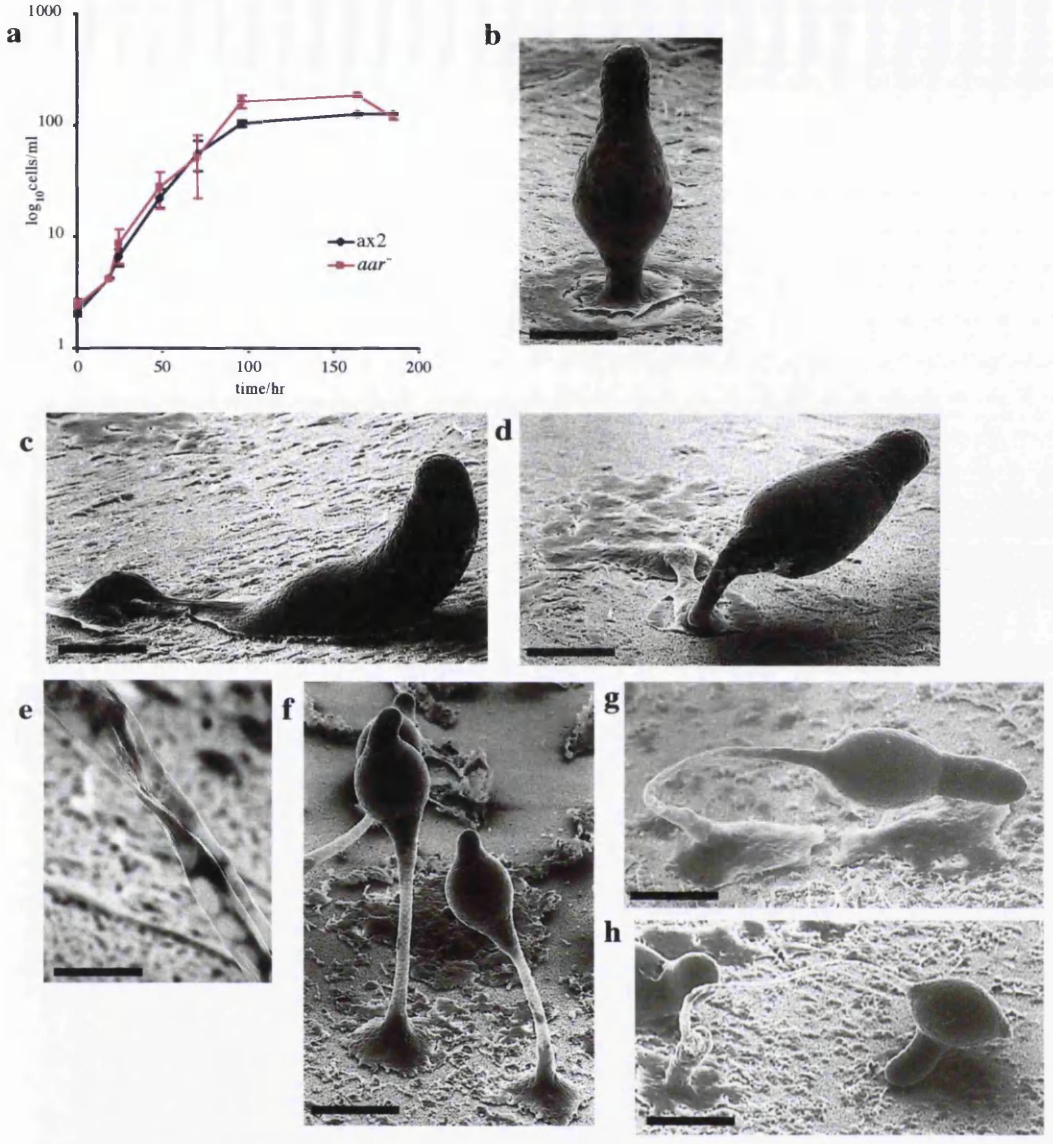


Figure 4.9 *aar*⁻ has morphological defects at culmination

a) *aar*⁻ grows at the same rate and to the same density as wild type in shaking culture. The experiment was performed three times, each time using three independent *aar*⁻ clones.

b) *aar*⁻ cells begin culmination normally.

c,d) *aar*⁻ stalks are weak and this causes the fruiting bodies to collapse and repeatedly attempt to raise their developing spore mass.

e) At high magnification (10x b-d) the stalk appears thin and contains few cells.

f) Wild type (AX2) culminants.

g) *aar*⁻ culminants have enlarged tips compared to wild type or ectopic tips (h) emanating from within the sorocarp.

Scale bar in b), c), d), g) and h) represents 50μm; in e) 10μm; in f) 100μm.

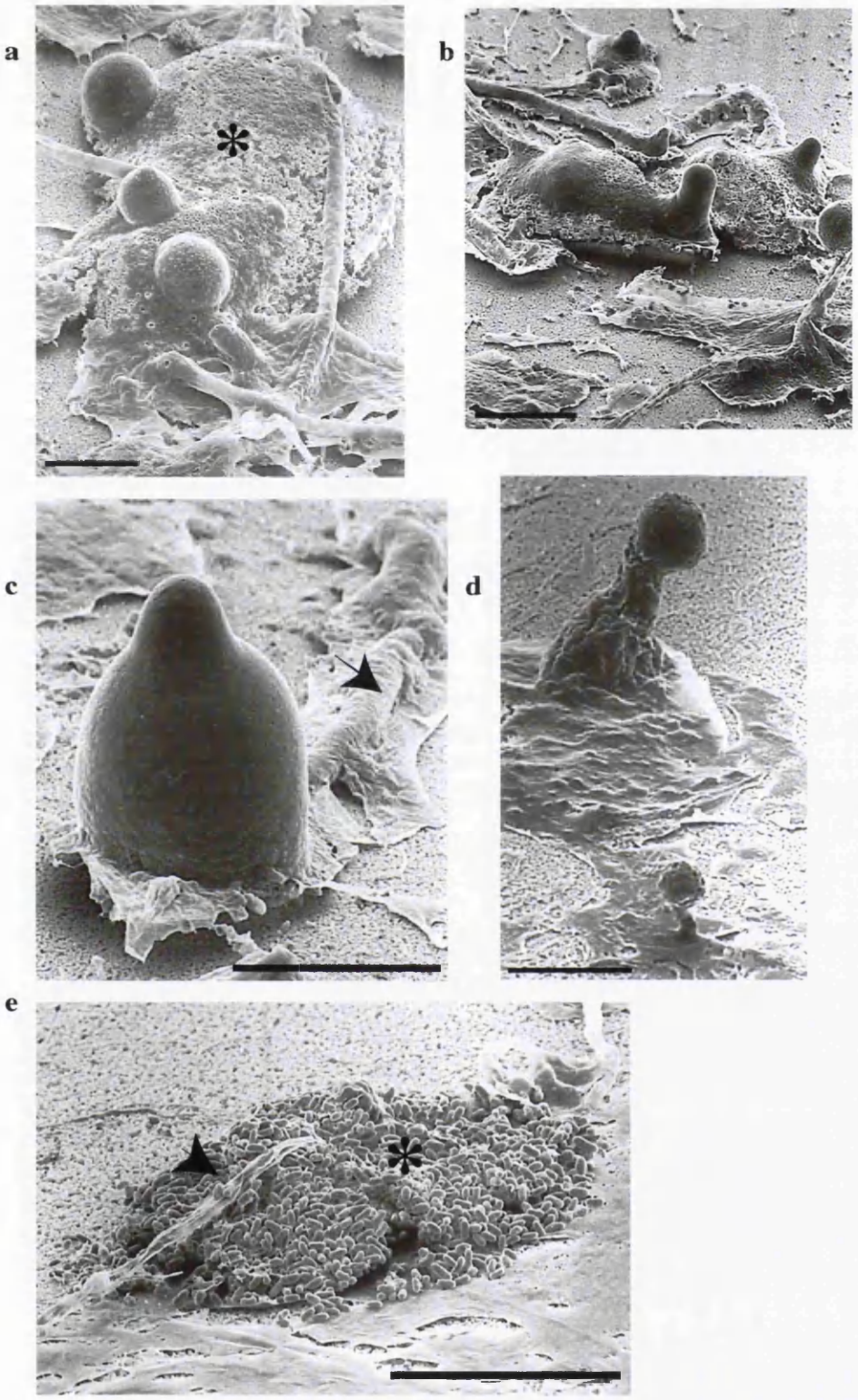


Figure 4.10 ***aar*⁻ forms reculminants from within the fallen fruiting body.**
Reculminant structures range from small outgrowths (a,b) to tipped mounds (c) to structures which attempt to make a "sporehead" (d).
e) Wild type fruiting bodies do not form reculminants, even if they collapse on the substratum.
The original spore mass is indicated with an asterisk in a) and e), a stalk by an arrow in c) and e). The scale bar represents 10μm in a) and d), 50μm in b), c) and e).

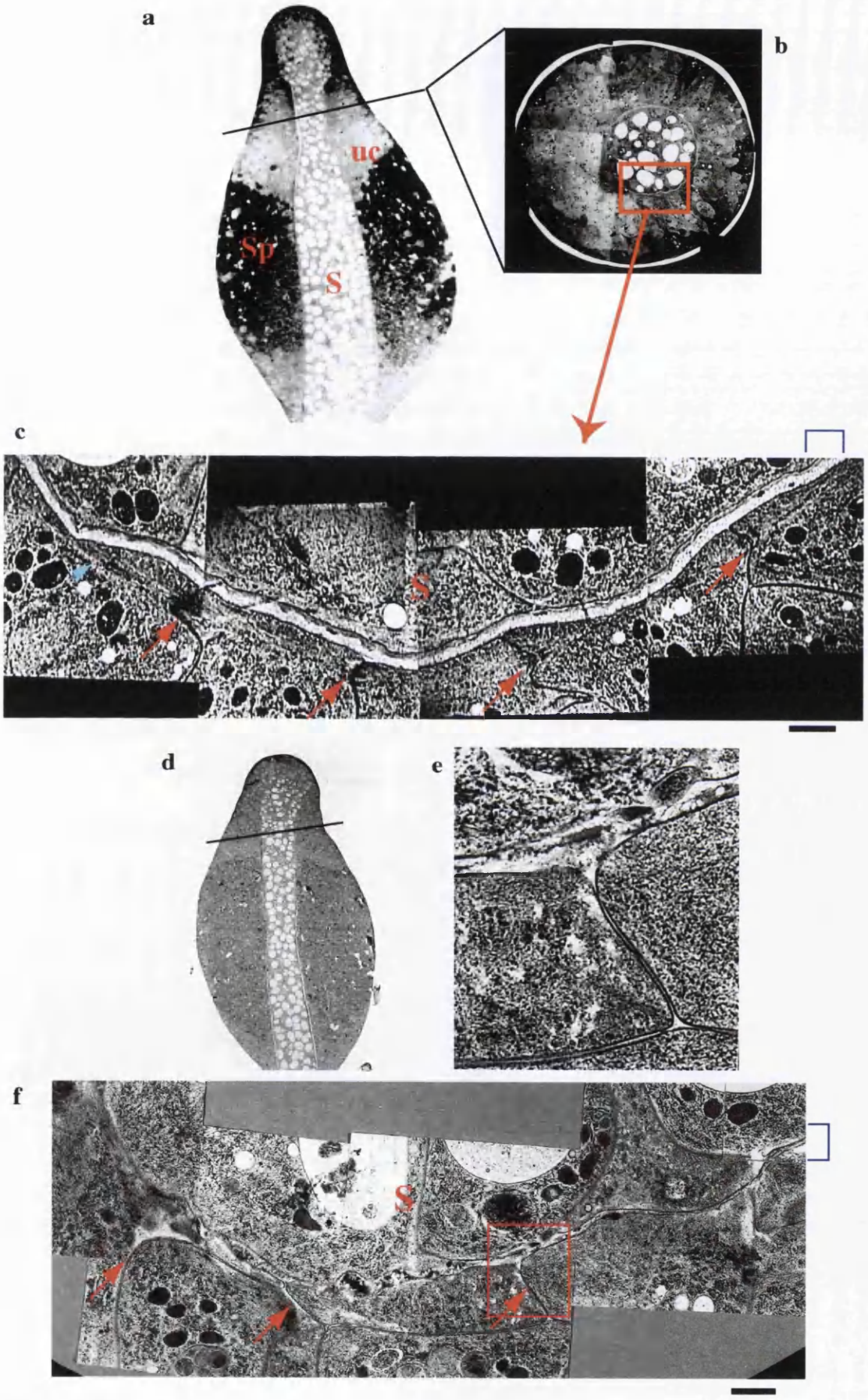


Figure 4.11 Please see the following page for legend.

Figure 4.11 *aar*⁻ mutants have no junctions

a) Median longitudinal section through a wild type (AX2) preculminant showing a constriction at the top of the stalk tube. The stalk itself (S) is 6-7 cells wide below the constriction and filled with vacuolated cells. The cells in the "funnel" above the constriction are prestalk cells which have not yet entered the stalk tube. The maturing spores (Sp) appear black. The lighter cells immediately above the spores are upper cup (uc) cells. The prestalk cells which abut the constriction, outside the stalk tube, contain junctions.

b) A cross section through the junctional region, marked by the black line in a). The vacuolated stalk cells in the stalk tube are clearly seen.

c) Higher magnification of part of this cross section shows junctions (red arrows) linked by a ring of actin filaments (light blue arrow) in the cells surrounding the stalk tube. Stalk cells (S) are separated from junctional cells by a distinct cellulose stalk tube bilayer (blue bracket).

d) Median longitudinal section through an *aar*⁻ mutant preculminant with an intact stalk tube. The stalk is only ~4 cells wide and no constriction is present.

e) The prestalk cells adjacent to the stalk tube do not contain junctions in the *aar*⁻ mutant (for a comparison with wild type see figure 3.5).

f) A cross section through the region in the *aar*⁻ mutant marked by the black line in d) shows that the stalk cells (S) are not encased in a double layer of cellulose (blue bracket) and cellulose deposition is thin and patchy. Surrounding prestalk cells do not contain junctions or an organised actin ring and are not polarised. The region shown in e) is marked by a red box. Red arrows show where the junctions would be in wild type cells.

Scale bars in c) and f) represent 1μm. e) is at three and a half times the magnification of f). The photographs in a)-c) were provided by M. J. Grimson.

4.7 Overexpression of Aar

4.7.1 Reintroduction of Aar into *aar*⁻ partly rescues the mutant morphology

In order to show that the culmination and structural defects observed in *aar*⁻ were specifically due to the absence of Aar protein, I attempted to rescue the phenotype by re-expressing Aar in mutant cells. A full-length *Kpn*I-*Xho*I *aar* cDNA PCR fragment was cloned into the multiple cloning site of pDXA-3C. This vector allows expression of protein throughout *Dictyostelium* development, under the control of the *actin-15* promoter ((Manstein *et al.*, 1995); figure 4.12). This cloning step added a C-terminal myc epitope to the Aar sequence at the expense of the final two amino acids of the Aar open reading frame. The vector was modified to allow protein expression using the ATG initiator codon in the *aar* sequence (R. Williams and A. J. Harwood, unpublished). The Aar-o/e (overexpression) construct was transformed into *aar*⁻ either alone, for random integration of the plasmid into the genome, or with the pREP plasmid to allow higher copy number extrachromosomal replication of the plasmid (Drury, 1996; Manstein *et al.*, 1995).

Transformants were developed on nitrocellulose filters: two clones produced robust fruiting bodies with fewer defective culminants, compared to *aar*⁻ transformed with empty vector (figure 4.13a,b,c). However, this was not a complete phenotypic rescue. Fruiting bodies were very small and some clones, including those transformed to high copy number with pREP, were indistinguishable from the untransformed *aar*⁻ mutant.

Importantly, *aar*⁻ cells transformed with Aar-o/e possess intracellular junctions. Unlike wild type junctions, Aar-o/e junctions are observed in both longitudinal and cross sections of preculminants. Instead of being discrete punctate structures, Aar-o/e junctions occupy large areas of the membrane and cause it to become buckled; aggregates of actin filaments and associated dense structures are observed (figure 4.13d). This shows that reintroduction of Aar protein into *aar*⁻ can rescue junction formation. Use of the *actin-15* promoter, which drives relatively strong expression of Aar throughout development, gives rise to transformants with an excess of junctions compared to wild type culminants. This suggests that the amount of Aar in wild type cells may be limiting for junctional formation. The apparent aberrant organisation of actin filaments into junctions by Aar-o/e could explain why full phenotypic rescue was not achieved. Misexpression of high levels of Aar could lead to a disruption of regulated cell-cell adhesion and morphogenetic cell movements at many stages of

development, which would perturb the structure of the final culminant. To investigate whether this was the case, Aar was expressed from the *actin-15* promoter in wild type (AX2) cells.

4.7.2 Expression of Aar-o/e in wild type cells leads to aberrant culmination.

Development of independent Aar-o/e expressing AX2 clones proceeds with normal timing and produces normal sized aggregates and first fingers. Overexpression of Aar-o/e in wild type cells appears to specifically affect culmination: the transformants form very small, stumpy fruiting bodies, compared to wild type cells transformed with vector alone. The stalks of culminants appear short and twisted and in many cases are unable to elevate the sporehead successfully (figure 4.14).

Thus either removal or overexpression of Aar results in fruiting bodies with a defect in stalk formation. In both cases fruiting bodies have weak stalks and tend to collapse. The small sporeheads of the Aar-o/e transformants could indicate a shedding of prespore cells during culmination in an attempt to raise the sporehead on a thin stalk, since earlier morphological stages are much larger.

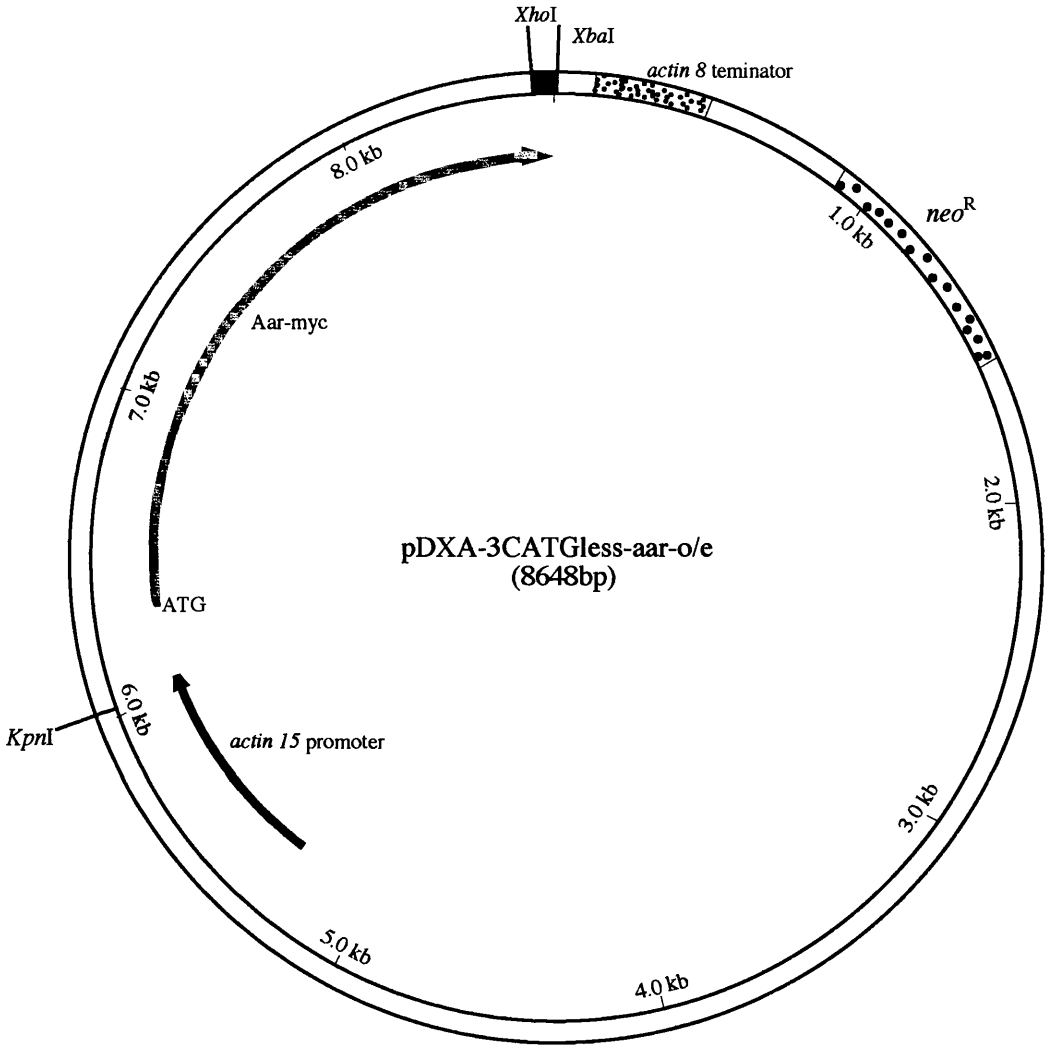


Figure 4.12 Construct used to express Aardvark in *Dictyostelium*.
The pDXA-3C vector was used to express full length Aar. A PCR fragment generated from the *aar* cDNA was cloned into the *Kpn*I and *Xho*I sites of pDXA-3C. The ATG translational start site downstream from the *actin 15* promoter in the vector was removed to allow use of the in-frame ATG in the Aar sequence.
The construct was provided by R. Williams and A. J. Harwood

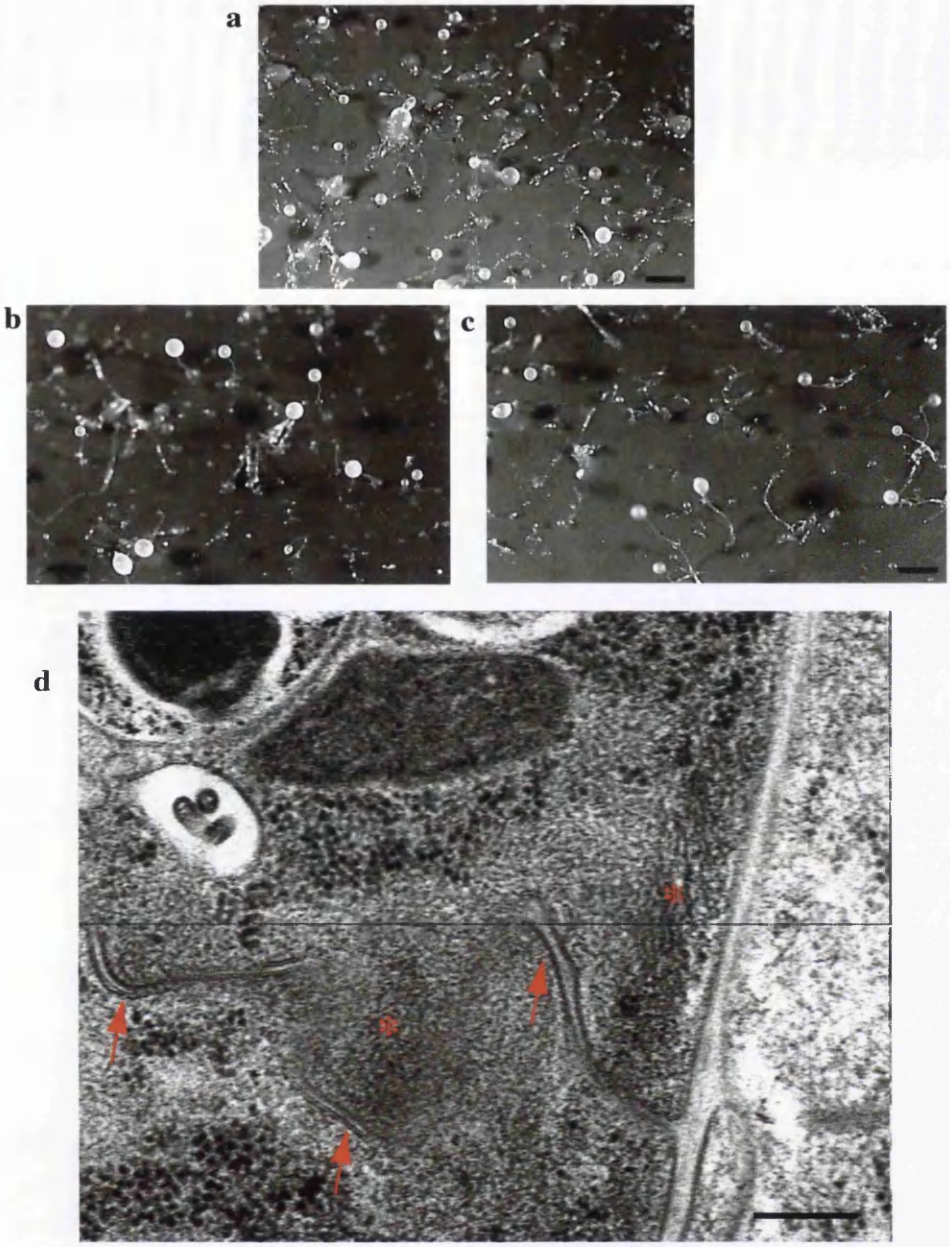


Figure 4.13 Overexpression of Aar in *aar*⁻ cells

a) *aar*⁻ mutants transfomed with a control vector-only construct form collapsed fruiting bodies after development on nitrocellulose filters for 24 hours.

b), c) 2 independent *aar*⁻ clones overexpressing Aar show increased numbers of standing fruiting bodies after 24 hours of development.

The scale bar in a)-c) represents 0.3mm.

d) *aar*⁻ mutants overexpressing Aar have large disorganised junctions (arrows). This is a longitudinal TEM section through a culminant from the transformants shown in b). Junctions are never observed in longitudinal sections of wild type culminants, only in cross sections (figures 3.5 and 4.11). The junction is associated with dense actin filaments (asterisks) and causes the plasma membranes of the adjoining prestalk cells to buckle. The dark granules are also associated with actin (M. J. Grimson, pers. commun.). The cellulose stalk tube is on the right. The scale bar represents 0.2μm.

The photograph in d) was provided by M. J. Grimson.

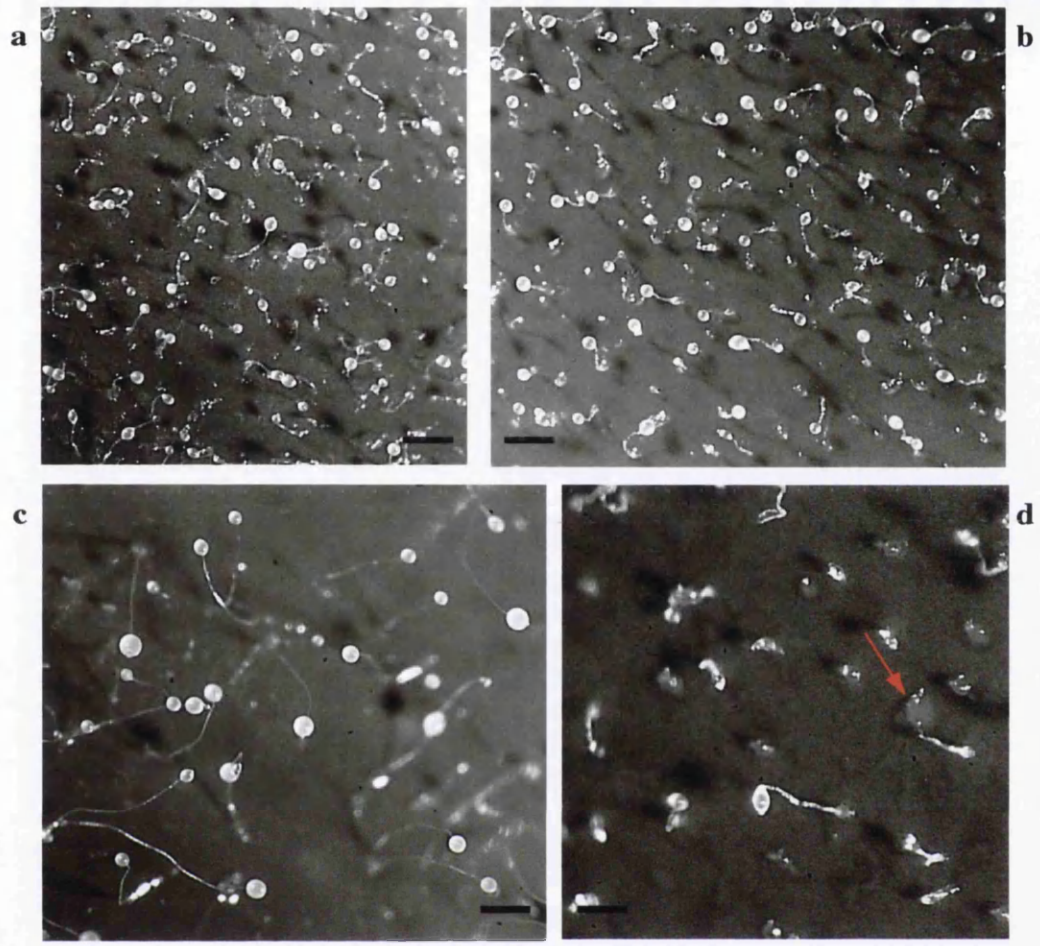


Figure 4.14 Overexpression of Aar in wild type cells

a), b) Two independent clones of wild type cells (AX2) overexpressing Aar produce very small fruiting bodies.

c) Control wild type cells transformed with vector only form wild type fruiting bodies.

d) Aar overexpressing culminants have thin, twisted stalks and fall over (arrow) like the *aar*$^-$ mutant.

The scale bars in a)-c) represent 0.5mm, in d) 0.2mm.

4.8 Generation of anti-Aardvark antiserum

4.8.1 Expression of a 6xHis-tagged Aar fusion protein in *E. coli*.

In order to investigate the localisation and regulation of Aar during development, a rabbit antiserum was raised to bacterially produced Aar protein. Two fragments of *aar* cDNA, encoding whole numbers of Arm repeats, were expressed using the pTrcHisB vector (Invitrogen). The advantages of this strategy were as follows:

- The 6xHis tag is small and non-antigenic (compared to, for example, GST) and does not need to be removed before immunisation.
- Purification of 6xHis tagged proteins is a relatively straightforward process due to their specific binding to metal affinity resins (in this study, Clontech Talon ™ was used).
- The vector used also added an epitope tag (Xpress tag, Invitrogen) which allowed the protein to be detected immunologically during the purification procedure.
- The tags were added at the amino terminus of the protein allowing single-step subcloning of the *aar* cDNA fragment into the vector. N-terminal tags have been successfully added to ß-catenin and plakoglobin previously, without affecting their intracellular localisation or binding properties (Aberle *et al.*, 1996; Fagotto *et al.*, 1996; Hulsken *et al.*, 1994; Miller and Moon, 1997; Molenaar *et al.*, 1996).
- The cDNA fragment used contained 22 nucleotides of sequence 3' to the Aar open reading frame for efficient translational termination and ease of subcloning.
- The fusion protein should fold correctly as a stable unit as a whole number of the repeats were expressed (Huber *et al.*, 1997a).
- Expression of Aar fusion proteins lacking the asparigine-rich N-terminus should prevent non-specific cross-reaction with other *Dictyostelium* proteins.
- The protein fragments chosen were small enough to be followed by SDS-PAGE during purification but not so large that they were likely to be degraded in the bacterial host (as often happens with full-length ß-catenin fusions, (V. Braga, pers. commun.; J. C., unpublished data).

- The 6xHis tag, unlike larger epitope tags, can bind to its cognate affinity resin under denaturing conditions (8M urea) allowing the purification of insoluble proteins.

Two expression constructs were generated in order to express 6xHis-Xpress tag-Aar fusion proteins (henceforth referred to as 6xHis-Aar) containing either ten or five Arm repeats. PCR primers were designed to introduce an *Xho*I site at the start of Aar repeat 1 or 6 (figure 4.15). The 3' ~1.5kb or ~0.75kb of *aar* cDNA was amplified using these primers with T7. The resulting PCR fragments were digested with *Xho*I and *Pst*I restriction enzymes (*Pst*I cuts within the pBluescript multiple cloning site at the 3' end of the fragment) and ligated into pTrcHisB, which confers ampicillin resistance on transformed bacteria. The constructs were transformed into *E. coli* DH5α. To discover the optimal conditions for protein expression, pilot inductions were performed with 10ml bacterial LB-ampicillin cultures. Protein expression was induced with 0.1mM, 0.5mM or 1mM IPTG at 37, 30, 27 or 22°C for different lengths of time (1 hour to overnight). Protein expression levels were low (<0.1μg/ml) under all conditions tried: no protein expression could be detected by Coomassie staining of whole cell lysates when compared to uninduced controls (data not shown). Expression of the five-repeat 6xHis-Aar fusion protein reached a maximum 1 hour after induction at 22°C (figure 4.16a). In addition, this five-repeat fusion protein was soluble in bacterial native lysis buffer (figure 4.16b), whereas the ten-repeat fusion was only soluble in 8M urea. Therefore the five-repeat 6xHis-Aar fusion protein was used for subsequent purification.

Figure 4.15 PCR primers used to amplify *aar* fragments for protein purification
The rpt 1X primer introduced an *Xho*I site at the start of repeat 1 and was used in conjunction with the T7 primer to amplify the entire repeat region of *aar* cDNA. The resulting PCR fragment was digested with *Xho*I and *Pst*I and ligated into pTrcHisB. The rpt 6X primer introduced an *Xho*I site between repeats 5 and 6 and was also used with the T7 primer to clone the last 5 repeats of Aar into pTrcHisB. The mismatches in the primers which introduced the *Xho*I sites into the sequence are shown in red.

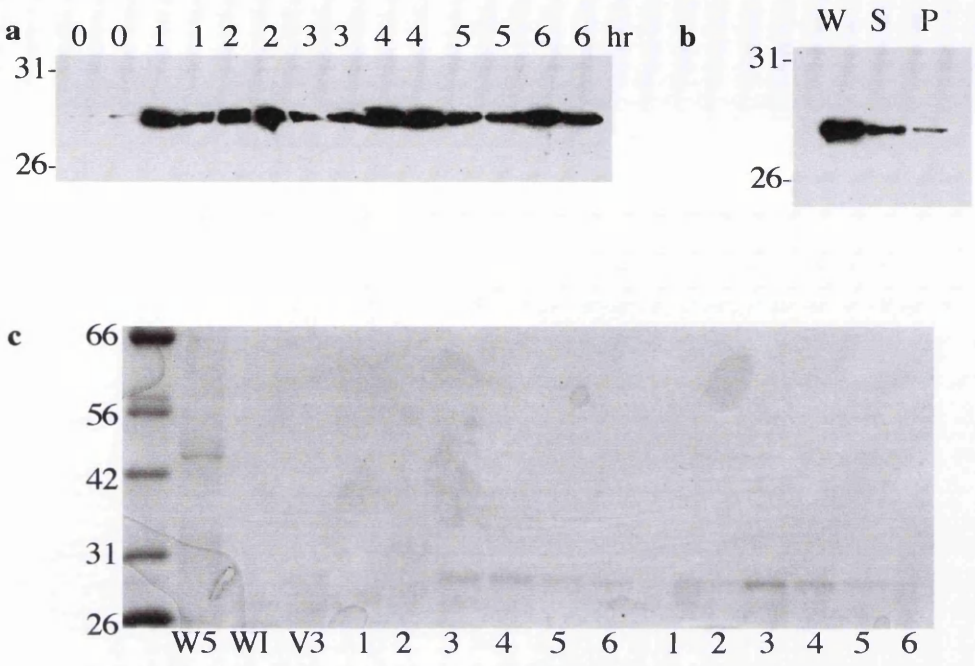


Figure 4.16 Bacterial expression and purification of an Aar protein fragment
a) Time course (0-6hr) of protein induction in two clones of 6xHis-Aar (last 5 repeats) transformants at 22°C, using 1mM IPTG. *E. coli* whole cell extracts were separated by SDS-PAGE and probed with anti-Xpress in a western blot. Expression did not increase further after 1 hour of induction in either clone so subsequent purification steps were carried out on bacteria induced for 1 hour.
b) 5-repeat 6xHis-Aar is largely soluble when bacteria induced at 22°C for 1 hour with 1mM IPTG are fractionated in native lysis buffer. Fractions were probed with anti-Xpress. W, whole cell extract; S, supernatant; P, pellet.
c) Coomassie-stained gel showing purification of soluble 5-repeat 6xHis-Aar. Molecular weight standards (New England Biolabs) are in the left hand lane: the 56, 42 and 31kDa bands each contain 1μg of protein. W5, pH5 column wash flowthrough; WI, 10mM imidazole column wash flowthrough; 1-6, first 6 fractions of a 75mM imidazole elution of pure Aar protein fragment (two independent purifications). V3, control elution, fraction 3, from a purification using vector-only transformants, showing that the protein eluted from 6xHis-Aar-expressing bacteria is specific.
Molecular weights (kDa) are shown on the left of each figure.

4.8.2 Loss of the 6xHis-Aar plasmid from bacteria

The low levels of 6xHis-Aar protein expression in *E. coli* led me to investigate whether the fusion proteins were toxic to bacteria. Cells transformed with the 6xHis-Aar (ten-repeat) plasmid were plated onto LB-agar containing 1mM IPTG, to force induction of protein expression. Freshly electroporated cells grow on IPTG to the same levels as on non-selective LB-agar (100%, table 4.1). This shows that IPTG is not toxic to bacteria *per se*, as most freshly electroporated cells are untransformed. However, bacteria grown overnight, in LB-ampicillin, from a single transformed colony, only grow to 1% of the non-selected level on IPTG plates (table 4.1). This shows that IPTG is toxic to cells that contain 6xHis-Aar plasmid and suggests that the 6xHis-Aar protein is toxic to the bacteria. However, these data do not preclude that the plasmid DNA itself could also be toxic.

If an expressed protein is toxic, then bacteria which eliminate or reduce protein expression by losing, or reducing the copy number of, the plasmid will have a selective advantage and outgrow high plasmid copy number bacteria in culture. Since low levels of protein expression can occur in uninduced cells (figure 4.16a), the selection pressure to eliminate protein expression may be present throughout the culturing procedure. To test whether the expression plasmid is lost over time, freshly transformed 6xHis-Aar (ten-repeat) bacteria, or those grown overnight in LB-ampicillin from a single transformed colony, were plated onto LB-agar with or without ampicillin.

Fresh 6xHis-Aar transformants grow on ampicillin to the same level as on non-selective agar (100%, table 4.1). 6xHis-Aar plasmid-containing bacteria grown overnight, from a single transformed colony, in LB-ampicillin give rise to slightly reduced numbers of colonies when plated on ampicillin. This suggests that the majority of cells retain the plasmid over time (table 4.1). Thus the low level of protein expression occurring in transformants grown in ampicillin may not be toxic to the cells, compared to the protein expression induced by 1mM IPTG, which kills ~99% of bacteria (table 4.1). Alternatively, the toxicity of even low-level protein expression may cause selection for bacteria containing very low copy numbers of plasmid when grown in ampicillin. This would explain why very little protein could be recovered from overnight cultures when they were induced with 1mM IPTG (section 4.8.1).

To test this possibility, bacteria were grown under more stringent antibiotic selection, to select for high copy number transformants. Ampicillin is degraded by the ß-lactamase enzyme produced by bacteria and, in addition, is susceptible to hydrolysis

in the acidic environment generated by bacterial metabolites. Thus bacteria with very low levels of plasmid (therefore at a selective advantage, due to protein toxicity) may be able to rapidly outgrow high plasmid copy number bacteria in overnight cultures, as the ampicillin is hydrolysed and its effective concentration in the culture reduced. Carbenicillin is an ampicillin analogue, which, although degraded by ß-lactamase, is resistant to hydrolysis. When a single 6xHis-Aar transformant colony was grown overnight in LB-carbenicillin and plated onto LB-agar containing carbenicillin, the number of colonies recovered was dramatically reduced, compared to the colonies obtained from non-selective plating or plating on LB-ampicillin (table 4.1). This shows that fewer bacteria survive if they are selected to contain high copy number 6xHis-Aar plasmid, using carbenicillin.

	No selection	IPTG	Amp	Carb
6xHis Aar fresh transformation 10µl	>1000 (100%)	>1000 (100%)	>1000 (100%)	>1000 (100%)
6xHis Aar overnight culture 10µl	1272 (100%)	16 (1.3%)	800 (63%)	51 (4%)
6xHis Aar overnight culture 100µl	4347 (100%)	53 (1.2%)	3780 (87%)	204 (5%)

Table 4.1 Plasmid stability assay. *E. coli* DH5α transformed with 6xHis-Aar (10 repeats) were plated on LB-agar containing no selection, 1mM IPTG, 50µg/ml ampicillin (amp) or 100µg/ml carbenicillin (carb). Freshly electroporated cells (10µl from 1ml) or transformants grown overnight in 10ml L-broth with amp or carb selection were plated overnight at 37°C. The recovered colonies counted and expressed as a percentage of the colony growth without selection (percentages in brackets).

Subsequent protein induction and purification was carried out using 100µg/ml carbenicillin selection. This ensured that all the bacteria present in the culture contained the highest possible plasmid copy number and would express maximal levels of protein when induced with IPTG.

4.8.3 Expression and purification of a soluble 6xHis-Aar protein fragment

Five-repeat 6xHis-Aar expression was induced for 1 hour at 22°C, in logarithmically growing bacteria. Supernatant from 1 litre of lysed culture was bound to Talon™ affinity resin (Clontech) and washes/elutions were performed using native lysis buffers of varying pH, or supplemented with imidazole. Imidazole allows elution of His-containing proteins away from the resin. His-tagged fusion proteins are expected to elute from Talon™ (Clontech) at pH6 (monomers) or pH5 (dimers). Attempts with various combinations of washes and elutions showed that the five-repeat 6xHis-Aar protein could not be eluted at pH5 or pH4, suggesting a tendency to aggregate on the

column. However extensive washing of the column at pH5, then with 10mM imidazole, removed all the background proteins and pure 6xHis-Aar was eluted with 75mM imidazole. Collection of small (250µl) eluate fractions allowed detection of the pure protein by Coomassie-staining (figure 4.16c). The estimated yield of protein obtained was around 25µg/litre of culture. The purification was repeated three more times, to produce enough protein for four inoculations of one rabbit. The rabbit chosen had pre-immune serum which did not cross react with *Dictyostelium* proteins, even at a 1:50 dilution, by immunofluorescence or western blotting (data not shown).

4.8.4 Anti-Aar antiserum recognises overexpressed Aar

A 10ml bleed taken from the rabbit one week after the fourth inoculation was tested at various dilutions on western blots of *Dictyostelium* whole cell extracts (figure 4.17). In vegetative cells overexpressing Aar, a protein of around 90kDa is detected. This is likely to be Aar-o/e, as untransformed vegetative cells do not contain this protein (figure 4.17b). The 90kDa protein is larger than the predicted size of Aar, but addition of C-terminal sequences in the construct may produce a band shift (see section 4.7). Interestingly, the antiserum did not detect Aar in wild type preculminant cells (figure 4.17a) although the *aar* message is maximally expressed at this time. This suggests that Aar levels may be under tight post-translational control, as seen for ß-catenin/plakoglobin. A small (~40kDa) protein is also present at high levels in Aar-o/e cells (figure 4.17a). It is interesting to speculate that this is a degradation product of Aar, caused by expressing Aar at the wrong time (vegetative cells) and to high levels.

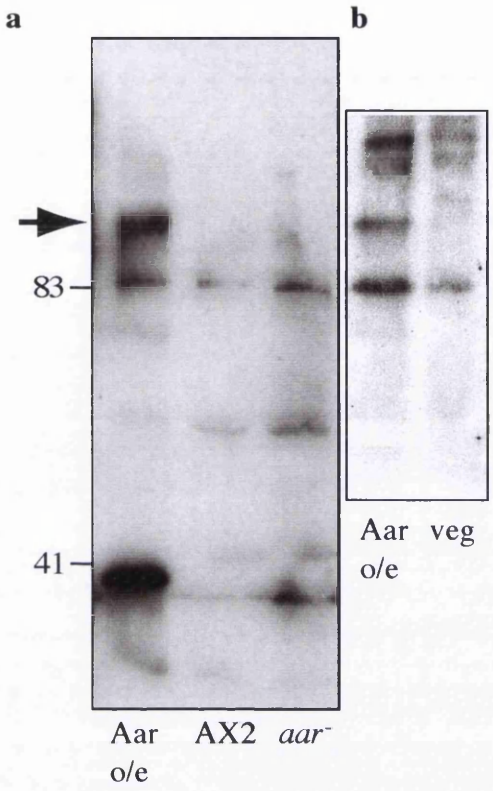


Figure 4.17 Anti-Aardvark recognises a specific protein in Aar-overexpressing cells.

Dictyostelium whole cell extracts from were separated by SDS-PAGE and probed with anti-Aardvark antiserum.

a) A ~90kDa protein is detected in vegetative cells transformed with Aar-o/e (arrow) which is not present in AX2 or *aar*⁻ preculminant extracts (AX2, *aar*⁻). Overexpressed Aar may be subject to degradation as a strong 40kDa band is also seen in Aar-o/e cells.

b) The ~90kDa band is absent from untransformed vegetative AX2 cells (compare veg with Aar o/e).

Figure 4.17b provided by E. Dalton.

4.9 Conclusions and discussion

4.9.1 Aardvark is a ß-catenin-like protein with a developmental function

I have cloned a *Dictyostelium* gene, *aardvark* (*aar*), which resembles Armadillo/ß-catenin/plakoglobin. *aar* encodes a protein with ten Arm repeats more similar in sequence to Arm and its homologues than to importins, p120 or smgGDS proteins, including those found in *Dictyostelium* (Vithalani *et al.*, 1998); M. Fukuzawa, pers. commun.; R. Williams, pers. commun.). The Aar protein sequence contains putative sites for gskA phosphorylation and may have an α-catenin binding region. Aar is necessary for the formation of intercellular junctions at the top of the stalk tube, which normally hold together an actin ring compressing the stalk tube in this area. The junctions and constriction are eliminated in the *aar*⁻ mutant. Possibly as a consequence of this, the cellulose stalk tube normally secreted by the developing stalk cells is severely disrupted, and *aar*⁻ culminants have weak stalks, which fail to elevate the sporehead. Analysing whether the presence of Aar mediates junction formation directly (by analogy with ß-catenin/plakoglobin) or by an indirect mechanism will require determination of the intracellular localisation of Aar.

4.9.2 Aar may be post-transcriptionally regulated

Although overexpressed Aar protein is clearly present in *Dictyostelium* cells, the antiserum raised to Aar does not detect endogenous Aar protein. This suggests that very little protein is present. However, *aar* message is expressed to a high level late in development, so Aar may be regulated post-transcriptionally, as is known to be the case for ß-catenin/plakoglobin. Interestingly, an antiserum raised to a *C. elegans* ß-catenin homologue, Bar-1, also only recognised overexpressed, not endogenous, protein (Eisenmann *et al.*, 1998). It may be possible to detect endogenous Aar by immunofluorescence on whole-mount developing structures. Alternatively, the antiserum may require affinity purification using the protein to which it was raised. Immunoabsorption of the antiserum against *aar*⁻ cells could also help to eliminate background. An ~80kDa background band is present in both wild type and *aar*⁻ cells which had not been detected by the preimmune serum, raising the possibility that anti-Aar might cross react with another related protein.

4.9.3 Problems with 6xHis-Aar purification

Why was Aar so difficult to purify from bacteria? Many other researchers have generated tagged versions of Arm proteins in bacteria; however, few publications give any indication of the yield of protein obtained. One of the few published examples of a bacterially expressed Arm protein is a full length *Drosophila* Armadillo-GST fusion protein (Peifer *et al.*, 1994b), figure 1). The signal detected in the bacterial extract using anti-Armadillo is very faint compared with the signal from *Drosophila* extracts and the authors do not state the yield of protein obtained, or the amount of bacterial extract required for purification. Full length GST-plakoglobin gave a very faint signal on a Coomassie-stained gel (Aberle *et al.*, 1996). Full length or nearly full length GST-Armadillo fusions were purified from *E. coli* DH5α, which undergo extensive degradation (Pai *et al.*, 1996). While this work was in progress a soluble MBP-full length Darlin (*Dictyostelium* smg GDS) fusion was purified and used for successful antibody generation, however no protein yield is stated (Vithalani *et al.*, 1998). The authors used 22°C induction in *E. coli* DH5α, under the control of a *tac* promoter similar to that in pTrcHisB. This suggests that the use of different bacterial strains or vectors would have been unlikely to increase my protein yield. The main difference between the two purification protocols was that MBP-Darlin expression was induced with 0.1mM IPTG overnight (Vithalani *et al.*, 1998), conditions that produced virtually no protein in my hands. Purification of His-tagged Vac8p-GFP (Fleckenstein *et al.*, 1998), using a similar promoter, but in bacteria which are designed to generate soluble proteins, was also successful for antibody generation but again no protein yield was given. The authors included detergent in the bacterial lysis buffer, which may have helped to eliminate protein aggregation problems, but column washes were carried out at pH 6.3 without detergent. It is not unreasonable to propose that the difficulties I encountered with purification are due to the aggregative nature of Arm proteins. The structure of these proteins (one assumes Aar is no exception) provides a surface for multiple protein-protein interactions (Huber *et al.*, 1997a). When concentrated, these superhelical proteins could perhaps interact with one another. Gel filtration analysis of cytosolic plakoglobin indicated its existence as a dimer *in vivo* (Cowin *et al.*, 1986) and high molecular weight aggregates of recombinant and endogenous ß-catenin have been detected in colon carcinoma cells (Stewart and Nelson, 1997). This type of aggregation could easily happen in bacteria, where high levels of protein will be expressed. This could prove toxic to bacteria and could lead to problems with column flow and elution during purification.

4.9.4 Conservation of Arm/ß-catenin functions outside metazoa

The sequence data together with the phenotype of the *aar*⁻ mutant suggest that Aar may be a functional homologue of ß-catenin/plakoglobin. This is the first report of such a homologue in a non-metazoan organism; and indeed the first report of adherens junctions outside metazoa (M. J. Grimson, pers. commun.). Therefore the functions of ß-catenin-like proteins seem to have arisen very early during the evolution of the multicellular state. Whether Aar, like ß-catenin, has a role in developmental signalling, in addition to a role in junctional formation, is investigated in the next chapter.

4.10 Appendix: cloning of a novel *Dictyostelium* gene in a degenerate oligonucleotide library screen.

To search for Armadillo/ß-catenin/plakoglobin homologues in *Dictyostelium*, a λZAPII library for 6-8 hours of development was probed at low stringency with a mixture of end-labelled degenerate oligonucleotides. The sequences of these oligonucleotides derive from regions of high homology between the known metazoan ß-catenin and plakoglobin genes (regions of amino acid identity in human ß-catenin and plakoglobin, mouse plakoglobin, *Drosophila* Arm, *Xenopus* plakoglobin and *Hydra* ß-catenin). The base composition was adjusted to reflect the *Dictyostelium* codon bias ((Sharp and Devine, 1989); Markus Maniak, pers. commun.) Three sets of degenerate primers were designed: the amino acid and deduced nucleotide sequences are shown in figure 4A.1.

An initial screen with an equimolar region of the "N-terminal repeat" oligonucleotides (figure 4.A1) yielded several positive colony pools, the inserts of which were amplified by PCR with T3 and T7 primers. A secondary screen was carried out on the pooled PCR products, which were separated on an agarose gel before blotting onto charged nylon membrane. The membrane was probed with the degenerate primer mix to identify positive inserts. One pool contained a positive insert in this screen: the cognate λ pool was replated at low density, to pick individual λ clones. Inserts from these clones were then reamplified by PCR and subjected to another round of blotting. A 900bp positive insert was obtained and sequenced from both ends, using T3 and T7 primers. A third internal sequencing primer was designed in order to cover the entire insert. The positive clone had no significant homology to Arm family proteins and encoded a novel *Dictyostelium* homologue of two yeast steroid isomerases, SRE1 and SUR4 (GenBank accession number AF187070). The coding sequence of this gene is shown in figure 4A.2.

Why did my oligonucleotides bind to the *Dictyostelium* SRE1-like sequence? It appears that the 5' end of the oligonucleotides used matched part of the clone sequence (figure 4A.2). It seems surprising that a match in half the oligonucleotide is sufficient to bind the clone DNA, but with the PCR-based secondary and tertiary screens a large amount of DNA is being probed, thus generating a strong signal.

N-terminal Armadillo repeats

```
    P   A   I   V   E   A   G   G   M   E
5' CCA GCW ATT GTW GAA GCW GGW GGW ATG CA  3'   (W=A or T)
5' CCA GCW ATC GTW GAA GCW GGW GGW ATG CA  3'
5' CCA GCW ATT GTW GAG GCW GGW GGW ATG CA  3'
5' CCA GCW ATC GTW GAG GCW GGW GGW ATG CA  3'
```

Nested Armadillo repeats

```
     V   Q   N   C   L   W   T
5'  GTW CAA AAT TGT TTA TGG AC  3'
5'  GTW CAA AAT TGT CTT TGG AC  3'
```

C-terminal region

```
     K   D   E   S   M*  R   F            * I in plakoglobin
3'  TTT ATC TTC WGA CAT TCT AAA  5'
3'  TTT ATC TTC WGA CAT TCT GAA  5'
3'  TTT ATC TTC WGA CAT ACG GAA  5'
3'  TTT ATC TTC WGA CAT ACG AAA  5'
```

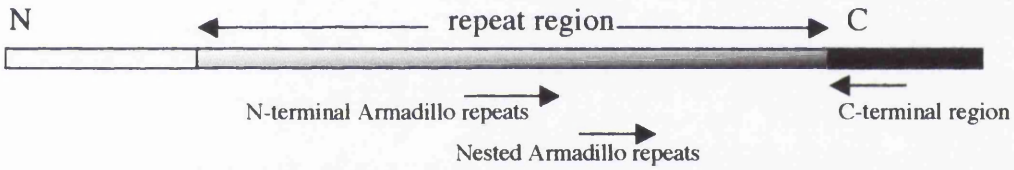


Figure 4A.1 Degenerate oligonucleotides designed to conserved regions of Armadillo, ß-catenin and plakoglobin. The "N-terminal Armadillo repeats" oligonucleotides lie within repeat 6. C-terminal oligonucleotides lie outside the repeat region.

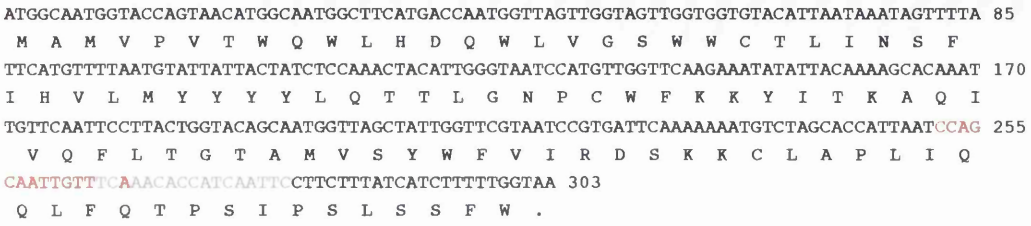


Figure 4A.2 Coding sequence of a *Dictyostelium* homologue of a yeast steroid isomerase.

The sequence in red denotes the residues which match that of the degenerate oligonucleotides used to screen a λ library, grey residues indicate mismatch with the oligonucleotides.

Chapter 5

A role for Aardvark in developmental signalling

5.1 Introduction

In addition to its role in intercellular adhesion, metazoan ß-catenin functions to transduce Wnt/Wg signals during development via its regulation by gsk3ß. As described in chapter 1, gsk3ß acts to antagonise the signalling functions of ß-catenin; during Wnt/Wg signalling gsk3ß activity is inhibited and ß-catenin is stabilised. The *Dictyostelium* homologue of gsk3ß, gskA, is required for the correct proportioning of prestalk and prespore cell types at the aggregate stage. During wild type development a subset of prestalk B cells (anterior-like cells), become the basal disc and lower cup in the fruiting body (Jermyn and Williams, 1991; Sternfeld and David, 1982). *gskA*$^-$ mutants have a highly expanded population of these prestalk B cells and a concomitant reduction in the prespore cell population. Thus gskA acts to repress prestalk B cell induction and promote prespore formation (Harwood *et al.*, 1995). Both these effects are mediated by a cAMP signal acting on the serpentine cAMP receptor, cAR3, which elevates gskA activity (Plyte *et al.*, 1999).

Dictyostelium amoebae can be induced to differentiate into stalk and spore cells in low density monolayer cultures, in the absence of cell contacts, by addition of the signalling molecules cAMP and DIF. This provides a simple system in which to study the developmental signal transduction pathways regulated by these molecules. DIF induces stalk cell formation in wild type cells (Berks and Kay, 1988; Berks and Kay, 1990; Harwood *et al.*, 1995; Jermyn *et al.*, 1987). Inclusion of cAMP with DIF inhibits stalk cell induction in low density monolayers (Berks and Kay, 1988; Berks and Kay, 1990; Harwood *et al.*, 1995). This effect of cAMP is mediated by cAR3 (Plyte *et al.*, 1999). *gskA*$^-$ amoebae readily form stalk cells in the presence of DIF. Unlike wild type, *gskA*$^-$ stalk cell induction cannot be inhibited by the inclusion of cAMP with DIF. Wild type cells can be induced to form spores in monolayer culture by incubation with 8-Bromo-cAMP. *gskA*$^-$ cells barely form spores under these conditions, due to their reduction in prespore cell differentiation (Harwood *et al.*, 1995).

Although gskA activity is regulated by cAR3, the downstream targets of gskA in this pathway are unknown. The similarity of Aar to ß-catenin and the presence of putative gskA phosphorylation sites within the Aar sequence led me to investigate whether the *gskA*$^-$ phenotype is caused by misregulation of Aar. I analysed the differentiation properties of isolated Aar cells to determine whether they had a cell-autonomous function in cell type specification. I also investigated the effects of Aar elimination or overexpression in the *gskA*$^-$ mutant.

5.2 *aar*⁻ mutants form reduced numbers of stalk and spore cells.

Logarithmically growing wild type (AX2) and *aar*⁻ amoebae were plated in low density monolayer culture ($2.9x10^4$ cells/cm^2) and induced to form stalk cells in the absence of any cell-cell contact. Amoebae were incubated for 24 hours in 5mM cAMP to render them competent to respond to DIF, and then for 24 hours with 0.1μM DIF, which induces terminal stalk cell differentiation under these conditions (Harwood *et al.*, 1995). In the second 24 hours some cells were incubated with a further 5mM cAMP in combination with DIF. In wild type cells this represses stalk cell differentiation in the monolayer (Harwood *et al.*, 1995). Stalk cell formation was scored as the percentage of round, vacuolated cells present after the second 24 hours.

Figure 5.1a shows that while wild type cells form around 70% stalk cells when incubated with DIF for 24 hours, *aar*⁻ cells form only about 45%, suggesting a defect in their differentiation. This is not simply due to a delay in terminal differentiation as the *aar*⁻ cells formed no further stalk cells after a further 24 hours in DIF (figure 5.1b). The difference between the mean number of stalk cells formed by wild type and *aar*⁻ cells is highly significant (0.001>P) in a two-tailed t-test. *aar*⁻ stalk cell formation is repressed to wild type basal levels by inclusion of 5mM cAMP with DIF (figure 5.1a,b). The difference between the mean number of stalk cells formed by wild type and mutant cells in the presence of cAMP and DIF is not significant (P>0.2). This suggests that the repression of stalk cell induction by cAMP, which is mediated by the pathway involving cAR3 and gskA (Harwood *et al.*, 1995; Plyte *et al.*, 1999), is normal in the *aar*⁻ mutant.

Various *aar*⁻ clones transformed with Aar-o/e were also tested for their ability to form stalk cells in the monolayer assay. High levels of Aar expression, achieved by cotransformation of Aar-o/e with pREP, rescue stalk cell induction to almost wild type levels while other Aar-o/e transformants show intermediate degrees of rescue (figure 5.1c). All transformants show wild type levels of repression of stalk cell formation by cAMP (figure 5.1c) suggesting that overexpressed Aar does not bypass the cAMP-mediated stalk cell repression pathway. This places Aar either downstream from, or in a separate pathway to, cAR3 and gskA during the regulation of stalk cell formation.

aar⁻ cells are also compromised in their ability to form spores in a monolayer assay (figure 5.1d). Spore cells were induced by incubating amoebae with 8-bromo cAMP (8-Br-cAMP) for 48 hours in low density culture ($1.5x10^3$ cells/cm^2). 8-Br-cAMP is a membrane-permeable analogue of cAMP which acts inside the cell to activate PKA and induce terminal spore differentiation (Harwood *et al.*, 1995; Hopper *et al.*, 1993b; Kay, 1989). Wild type cells form approximately 10% spores in the

monolayer assay; *aar*⁻ cells formed only half this number. The difference between the mean number of spores formed by wild type and *aar*⁻ cells is highly significant (0.001>P). Interestingly, experiments with spores isolated from mature fruiting bodies show that *aar*⁻ spores are only half as viable as wild type spores (figure 5.1e; 0.05>P>0.02) when plated onto SM/*Klebsiella* and left to germinate. The two-fold reduction in spore cell formation in *aar*⁻ is less severe than the twenty five-fold reduction observed in *gskA*⁻ (Harwood *et al.*, 1995) suggesting that loss of Aar activity is not the sole cause of the *gskA*⁻ spore induction defect.

These data show that the *aar*⁻ mutant is defective in stalk and spore cell differentiation. How do these monolayer culture results relate to what is seen in the developing fruiting body? Does the *aar*⁻ mutant have a defect in the specification of prespore and prestalk cell types early during development (as does the *gskA*⁻ mutant) or in the terminal differentiation of prespore and prestalk cells? The morphology of the *aar*⁻ mutant shows that *aar*⁻ cells can form a stalk and a spore mass with approximately correct proportions. This is unlike the *gskA*⁻ mutant where less than 1% of cells form spores, compared to 80% of cells in wild type culminants. To analyse whether *aar*⁻ cells can form prestalk and prespore cell types normally, their developmental expression of markers for prestalk A and B and prespore cell types was analysed by northern blotting.

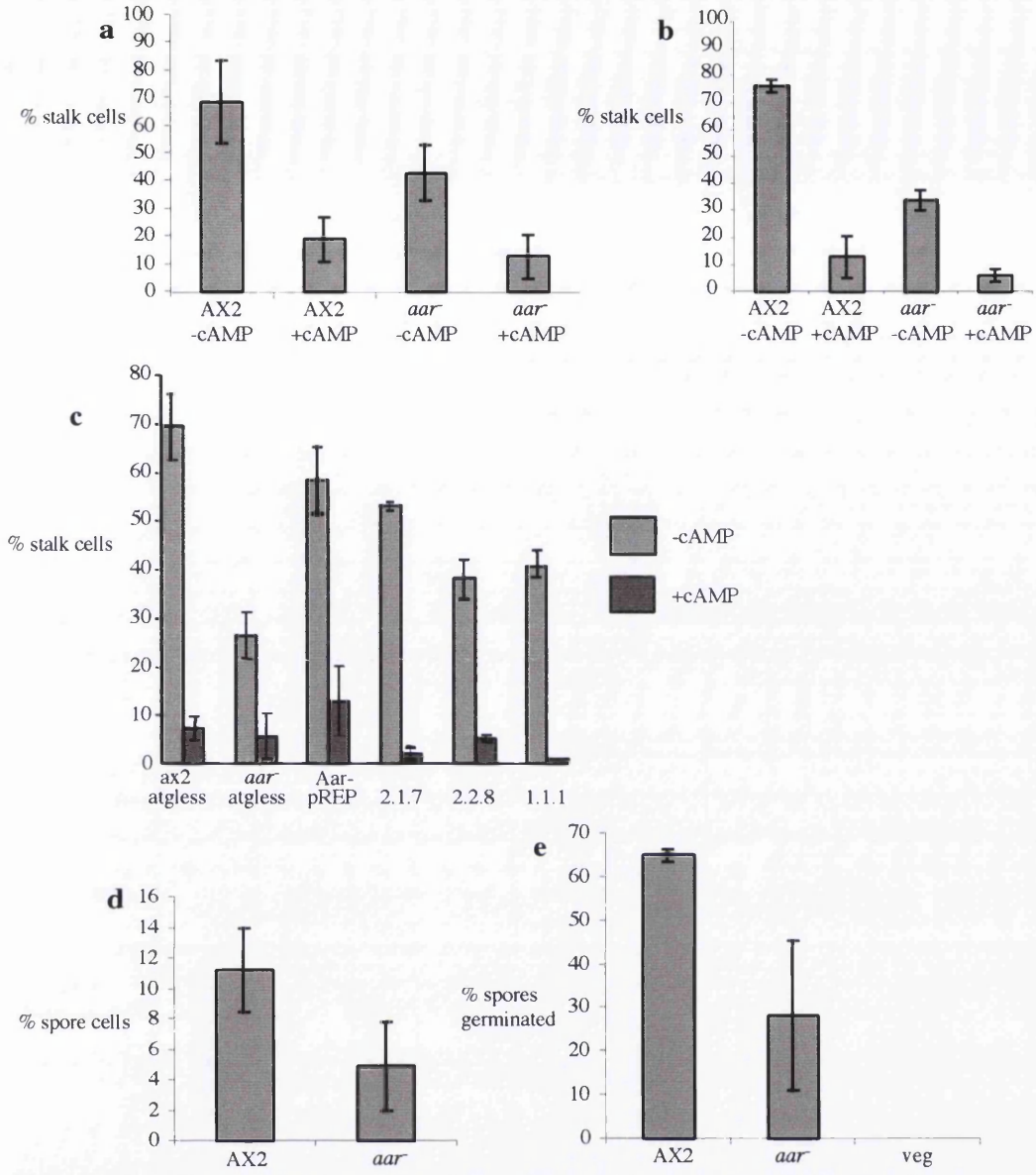


Figure 5.1 The *aar*⁻ mutant has defects in stalk and spore differentiation.

a) Stalk cell monolayer assay. In the absence of cAMP, wild type (AX2) cells form ~70% stalk cells when incubated with DIF for 24 hours, *aar*⁻ form only ~45%. Two independent mutant clones were tested in four experiments, each in triplicate. Stalk cell induction is inhibited by cAMP in both cell types.

b) Stalk cell monolayer assay incubated with DIF in the presence or absence of cAMP for 48 hours. *aar*⁻ does not form wild type numbers of stalk cells even after this period of incubation, indicating that it is not simply slower to form stalk cells than wild type.

c) Aar-o/e rescues stalk cell induction in *aar*⁻. Two independent monolayer assays were performed, each in triplicate, on AX2 or *aar*⁻ cells transformed with a vector-only control (AX2/*aar*-atgless) and on four Aar overexpressing *aar*⁻ clones (Aar-pREP, 2.1.7, 2.2.8, 1.1.1). The overexpressing clones show different degrees of rescue. All the transformants show inhibition of stalk cell induction in the presence of cAMP.

d) Spore monolayer assay. *aar*⁻ forms half the wild type number of spores. Two independent mutant clones were tested in three experiments, each in triplicate.

e) *aar*⁻ spores isolated by detergent treatment of fruiting bodies have reduced viability when germinated on SM/*Klebsiella*. Vegetative cells (veg) do not germinate after detergent treatment. Three independent mutant clones were tested and the experiment was performed in duplicate.

5.3 Expression of cell type specific mRNAs in the *aar*⁻ mutant

mRNA from various stages of development was tested for the expression of the *ecmA*, *ecmB* and *psA* genes, which are specific to prestalk A, prestalk B and prespore cells, respectively (Early *et al.*, 1993; Early *et al.*, 1988; Jermyn *et al.*, 1989; Jermyn and Williams, 1991). *aar*⁻ mutants express all three markers and are thus able to form prestalk A and B and prespore cells during development (figure 5.2) Induction of *psA* is delayed by around 2 hours in the *aar*⁻ mutant compared to wild type. This correlates with a delay in development observed: *aar*⁻ cells are slow to aggregate (2-3 hours behind wild type) and once mounds have formed development proceeds with almost normal timing; wild type cells reach mid-culminant 18-19 hours post-starvation while *aar*⁻ cells take 22 hours. Overexpression of Aar does not rescue this developmental delay, which may therefore not be specifically due to the disruption of *aar*. The level of *psA* mRNA is reduced at least 2-fold in the *aar*⁻ mutant (figure 5.2), indicating a possible defect in prespore cell induction. Levels of *ecmB* mRNA are also lower in *aar*⁻ than in wild type, particularly late in development (figure 5.2). Only a small proportion of wild type cells express *ecmB* before culmination begins (Jermyn *et al.*, 1989; Jermyn and Williams, 1991) but as prestalk A cells in the tip of the slug enter the stalk tube they begin to express stalk-specific *ecmB* (Ceccarelli *et al.*, 1991; Jermyn and Williams, 1991). The reduced level of *ecmB* expression in *aar*⁻ cells could therefore be due to a reduced number of prestalk A cells entering the stalk tube and turning on stalk-specific *ecmB* gene expression. *aar*⁻ induces *ecmA* several hours after wild type cells (figure 5.2) but peak expression is comparable to that observed in the wild type.

These data indicate that the inability of *aar*⁻ to make wild type numbers of mature stalk and spore cells could be due to a defect in prestalk and prespore cell formation. Unlike *aar*⁻ cells, *gskA*⁻ cells show elevated *ecmB* expression at the mound stage and negligible prespore expression (Harwood *et al.*, 1995), showing that the *gskA*⁻ phenotype is not solely brought about by misregulation of Aar. The data do not preclude that the reduction in prespore/spore formation observed in *gskA*⁻ cells could in part be mediated by loss of *aar*⁻, or that the upregulation of *ecmB* in *gskA*⁻ cells could be due to an overproduction of Aar. However, gskA must have other downstream targets, besides Aar, if this is the case.

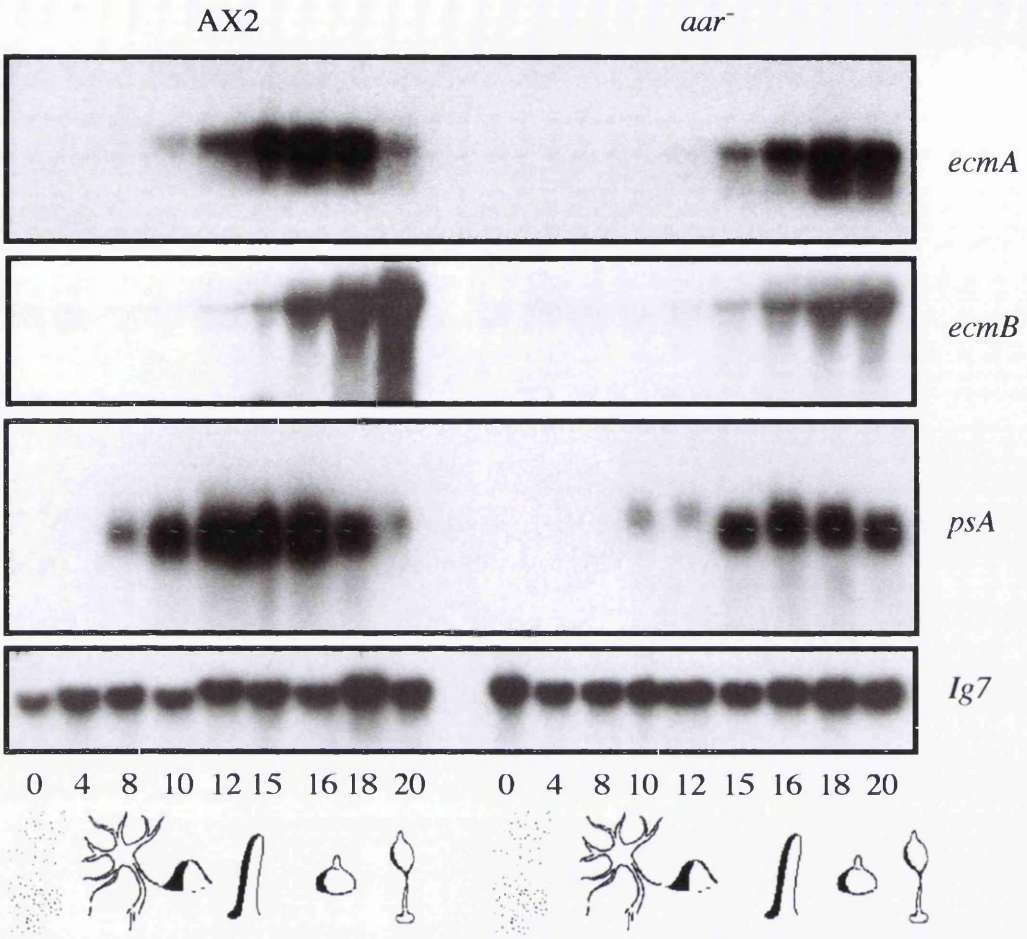


Figure 5.2 *aar*⁻ expresses prespore and prestalk-specific genes
Northern blotting of total RNA from various stages of development shows that the *aar*⁻ mutant expresses the prestalk-specific genes *ecmA* and *ecmB* and the prespore-specific gene *psA*. Expression of both *ecmA* and *psA* is delayed in the *aar*⁻ mutant compared with wild type (AX2). Levels of*ecmB* and *psA* are reduced in *aar*⁻. The probes for *ecmA*, *ecmB* and *psA* are PCR products and that for the *Ig7* loading control is plasmid DNA (all provided by E. Dalton).

5.4 The genetic relationship between *aar* and *gskA*

Is *aar* genetically downstream of *gskA*? If so, one prediction would be that an *aar⁻/gskA⁻* double mutant would have reduced ability to form mature stalk cells in a monolayer. If the excess of prestalk B cells in the *gskA⁻* mutant was caused by upregulation of Aar (as predicted from the metazoan model) then an *aar⁻/gskA⁻* double mutant might have a reduced prestalk B population *in vivo*. Likewise overexpression of Aar in the *gskA* mutant might exaggerate the *gskA⁻* phenotype or at least have no further effect. However, these predictions turned out to be incorrect.

5.4.1 The *aar⁻/gskA⁻* mutant resembles the *gskA⁻* mutant

Independent *aar⁻/gskA⁻* mutant strains were generated by homologous recombination of a blasticidin-resistant *aar* knockout construct into *gskA⁻* cells. The disruption of the *aar* gene was confirmed by Southern blotting of EcoRV-digested genomic DNA with an *aar* cDNA probe and by an inability to amplify an internal *aar* PCR product from genomic DNA (figure 5.3a,b). Three different clones were chosen for further characterisation. These three clones have slightly different morphology when developed on SM/*Klebsiella* as determined in the initial screen for *aar⁻/gskA⁻* transformants. Clone 3.4.5 forms structures with thick bases and occasional small stalk-like protrusions as seen in *gskA⁻*. Clones 2.3.3 and 2.3.5 form small mounds with no protrusions (figure 5.3c). However when developed on nitrocellulose filters (or KK_2 agar, data not shown) all three knockout clones resembled the *gskA⁻* mutant (figure 5.3c). Terminal structures formed by the *gskA⁻* or *aar⁻/gskA⁻* mutants stain identically with tinopal, which marks the presence of cellulose, indicating all are able to form mature stalk cells (data not shown). This was corroborated by a monolayer assay showing that all the double mutant clones, like *gskA⁻*, produced between 90 and 100% stalk cells with DIF, which could not be inhibited by inclusion of cAMP in the assay (figure 5.3d).

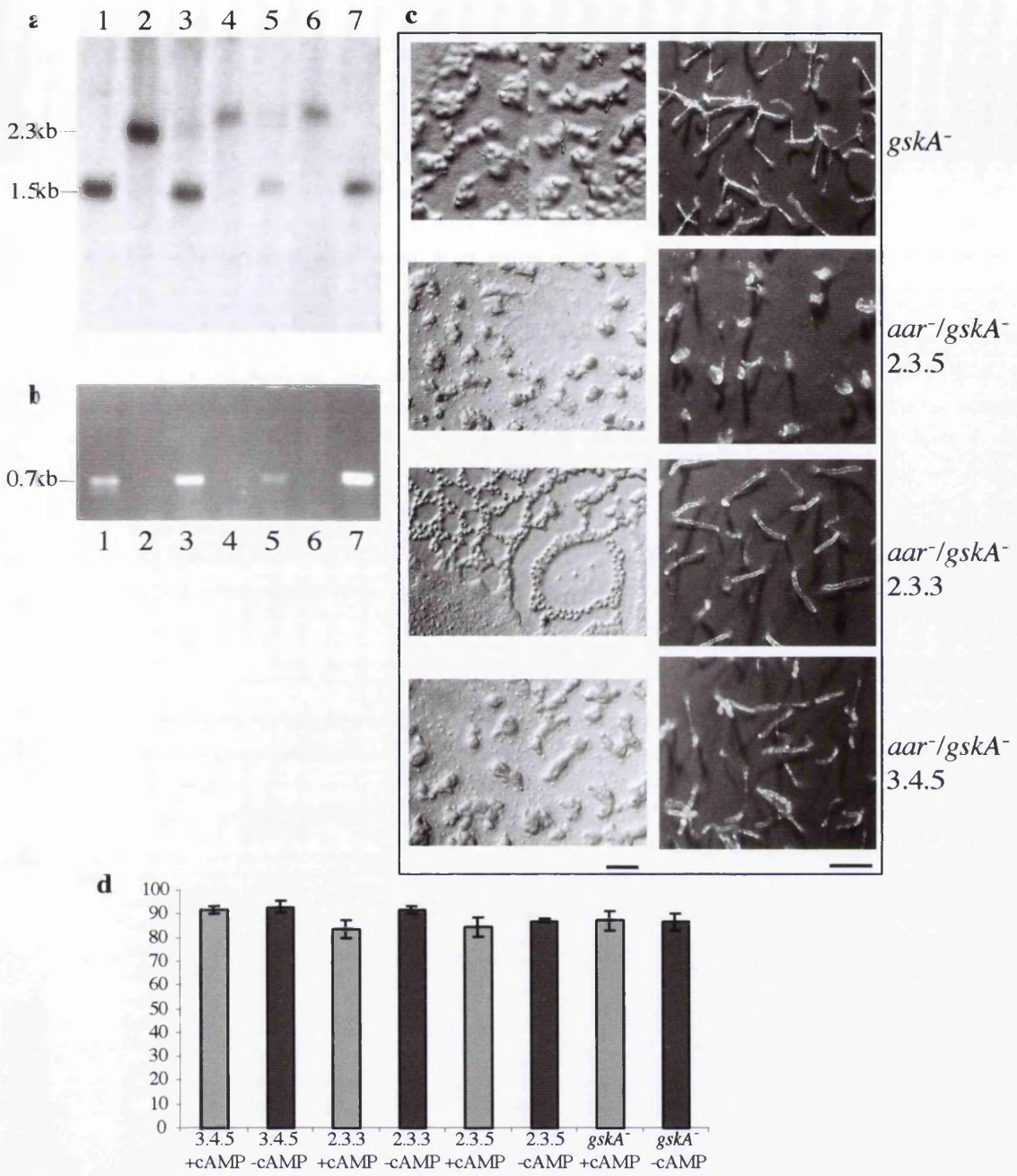


Figure 5.3 Disruption of *aar* in a *gskA*⁻ background.

a), b) *gskA*⁻ cells were transformed with the blasticidin-resistant *aar* knockout construct used in chapter 4. Genomic DNA was digested with EcoRV for Southern blotting (a) or amplified by genomic PCR (b) with internal *aar* primers. Lane 1: *gskA*⁻, Lanes 2,4,6: *aar*⁻/*gskA*⁻ (three independent clones), lanes 3,5,7: incorrect integration of the knockout construct into the *gskA*⁻ genome.

c) The three *aar*⁻/*gskA*⁻ clones were developed on SM/*Klebsiella* (left hand panels) or nitrocellulose filters (right hand panels). While clone 3.4.5 resembles *gskA*⁻ on SM/*Klebsiella*, clone 2.3.3 forms small mounds and clone 2.3.5 forms mounds with a characteristic clustered pattern. However, on filters clones 2.3.5 and 3.4.5 developed indistinguishably from *gskA*⁻ and 2.3.3 forms short thick *gskA*-like structures with no sporehead. The scale bars represent 1mm.

d) All three double knockout clones behave identically to *gskA*⁻ in a monolayer, forming over 90% stalk cells in the absence or presence of cAMP.

The simplest interpretation of this result is that Aar functions independently of gskA. It is possible that Aar is only required for the terminal differentiation of the subset of prestalk cells, the A and AB cells, which eventually enter the stalk tube (Jermyn and Williams, 1991). Since the *gskA*⁻ mutant specifically overproduces prestalk B cells, most of which eventually differentiate to form the basal disc and upper and lower cup, the phenotype of the double knockout would be largely *gskA*-like. Any A/AB cells present in the *gskA*⁻ mutant, which were missing in the *aar*⁻/*gskA*⁻ mutant, could have been overlooked. The comparison of expression of *ecmA* and stalk-specific *ecmB* in the mutants would be required in the future to resolve this issue. In this respect it is of note that two *aar*⁻/*gskA*⁻ clones did not form stalk-like protrusions on SM/*Klebsiella* (figure 5.3).

Although *aar* is maximally expressed a long time after gskA has acted to regulate prestalk/prespore proportioning, these results do not preclude that either a) Aar may be regulated by gskA later in development, or that b) Aar may act downstream of gskA early in development but is functionally redundant with another Aar-like protein. However, the consequences of Aar overexpression in a *gskA*⁻ background suggest that Aar is capable of affecting early development.

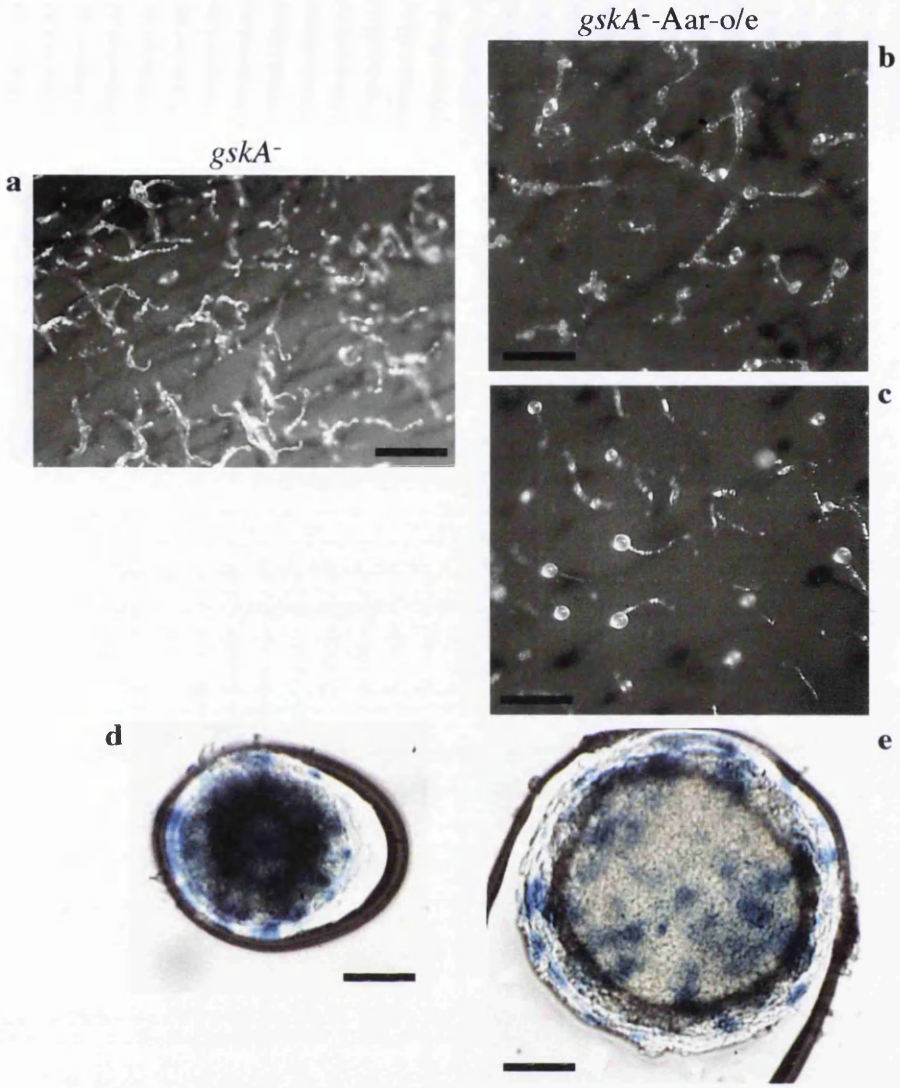
5.4.2 Overexpression of Aar in the *gskA*⁻ mutant partly rescues the *gskA*⁻ phenotype.

Aar-o/e was transformed, alone or in conjunction with pREP, into *gskA*⁻ cells. Several independent transformant clones made small structures that resemble wild-type fruiting bodies, suggesting that overexpressed Aar rescues the *gskA*⁻ morphological defect (figure 5.4a-c). Is Aar acting to reduce the prestalk B cell population of the *gskA*⁻ mutant to a wild type level during development? To address this possibility *gskA*⁻ cells were cotransformed with Aar-o/e or control empty vector, and *ecmB-lacZ*, a stable reporter for the prestalk B cell population. It was assumed that any G418-resistant cells obtained would contain both DNA constructs. Observation of the *gskA*⁻ mutant (vector-only transformants) at the mound stage revealed that most of the cells in the mound express *ecmB* (figure 5.4d) as described previously (Harwood *et al.*, 1995). Aar-overexpressing mounds show greatly reduced *ecmB-lacZ* expression (figure 5.4e) suggesting that the presence of Aar-o/e reduces the proportion of prestalk B cells in the aggregate. This could explain the more wild type morphology of the culminants. The nature of the non-*ecmB* expressing cells in the aggregates was not investigated. Cotransforming Aar-o/e with *psA-lacZ* would be required to confirm whether they were

prespore cells. All the transformants lacked the cAMP-mediated repression of stalk cell formation in a monolayer (figure 5.4f). Therefore under these conditions Aar cannot prevent the overproduction of prestalk/stalk cells by *gskA*⁻ in the presence of cAMP. However the monolayer is an artificial system where early developmental signalling events are bypassed by the presence of millimolar levels of cAMP in the first 24 hours of the assay. In addition the cAMP-insensitive stalk cells formed may not be prestalk B cells.

5.5 Upregulation of Ddplako in the *aar*⁻ mutant

The fact that *aar* is maximally expressed long after the prestalkB:prespore specification by gskA has occurred suggests strongly that the gskA does not downregulate Aar in order to inhibit prestalk B formation *in vivo*. Moreover overexpression of Aar can rescue some aspects of the *gskA*⁻ phenotype. Thus the regulation of Aar is not analogous to the downregulation of ß-catenin by gsk3ß in this situation. Why would the presence, but not the absence, of Aar affect the development of the *gskA*⁻ mutant? One explanation is that Aar does not usually function downstream of gskA during prestalk B:prespore patterning but that a related molecule does. If an Aar-like protein was normally upregulated by gskA activity in order to inhibit prestalk B formation then its function would be reduced in the *gskA*⁻ mutant. Ddplako seems an obvious candidate for such an Aar-related protein: it is expressed during early development and its expression is delayed and reduced in the *gskA*⁻ mutant. Overexpression of Aar early in development could be substituting for a lack of Ddplako (or other Aar-related protein) and hence rescuing the *gskA*⁻ defect. Interestingly, Ddplako consistently appears to be elevated approximately two-fold in *aar*⁻ cells (figure 5.5), suggesting that upregulation of Ddplako is occurring in response to loss of Aar.



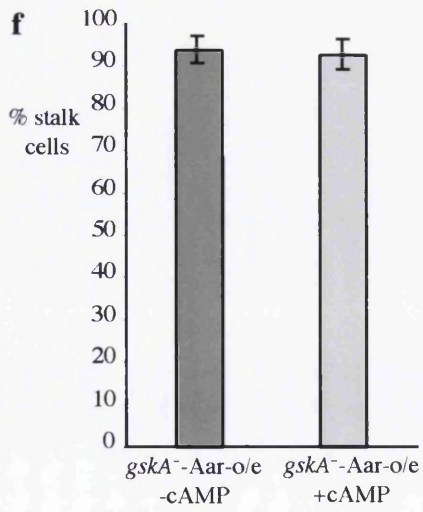


Figure 5.4 Overexpression of Aar in *gskA*⁻ rescues its morphological defect.
a) *gskA*⁻ cells transformed with a vector-only control and developed for 24 hours on filters show a *gskA*⁻ morphology.
b), c) Two independent Aar-o/e transformants developed for 24 hours show wild type rather than *gskA*⁻ morphology, having a discernible sporehead and stalk. However the clone in (b) has thick bases reminiscent of *gskA*⁻. The scale bars in a)-c) represent 0.5mm.
d) *gskA*⁻ mound expressing *ecmB-lacZ*.
e) Aar-o/e transformant with reduced *ecmB-lacZ* expression at the mound stage. The scale bars in d) and e) represent 0.1mm.
f) Aar overexpressing *gskA*⁻ cells form stalk cells in the presence or absence of cAMP in monolayer culture and resemble *gskA*⁻ in this respect.

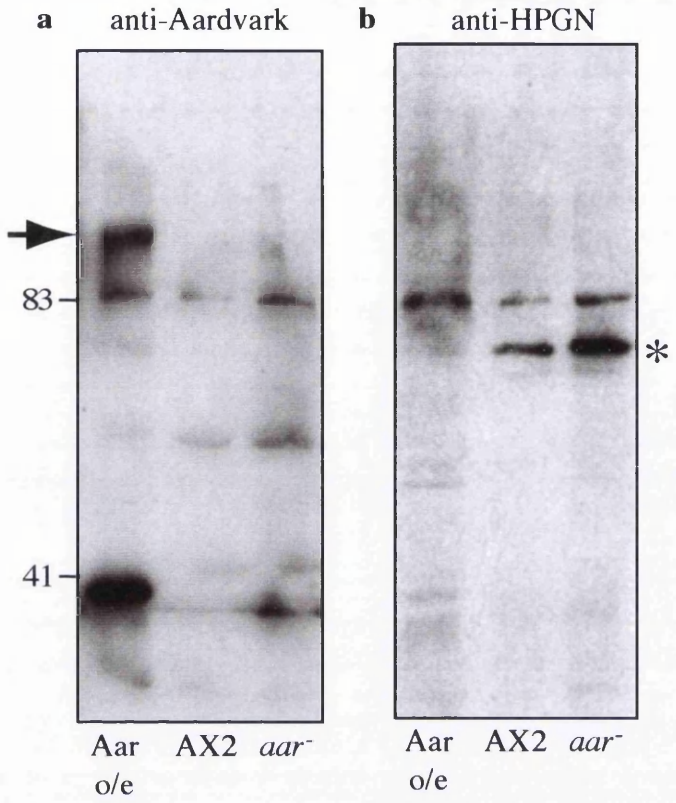


Figure 5.5 Ddplako is upregulated in *aar*⁻ cells.
Dictyostelium whole cell extracts from vegetative cells overexpressing Aar (Aar o/e), untransformed wild type preculminant cells (AX2), or untransformed *aar*⁻ preculminant cells (*aar*⁻) were separated by SDS-PAGE.
a) Anti-Aardvark antiserum detects overexpressed Aar (arrow) in vegetative cells but not endogeneous Aar in wild type preculminants. This figure was previously shown as figure 4.16a.
b) An identical blot was probed with anti-HPGN, which recognises Ddplako (*). Ddplako is expressed at a higher level in the *aar*⁻ mutant than in wild type cells. The presence of the 83kDa band in both blots is not understood.

5.6 Conclusions

The inability of *aar*⁻ cells to form wild type numbers of stalk or spore cells in monolayers demonstrates that Aar has a cell-autonomous role in differentiation in addition to its function in intercellular adhesion. This correlates well with the thin stalks, containing fewer stalk cells than wild type, observed in the *aar*⁻ mutant (figure 4.9, 4.11). However these effects could also be explained by the inability of prestalk cells to pass into the stalk tube and terminally differentiate in the absence of intercellular junctions. In addition, prestalk or prespore cells that do not terminally differentiate may be the cells that instead form reculminants. Wild type fruiting bodies do not form reculminants even when lying on the substratum, presumably because all cells have terminally differentiated by this time. Moreover, Aar-o/e fruiting bodies do not form reculminant structures. *aar*⁻ reculminants appear to stain strongly with the prestalk-specific markers *ecmA-lacZ* and *ecmB-lacZ*, however these structures are fragile and difficult to fix. This staining indicates that these cells were, at least once, prestalk cells. Interestingly, structures that may be similar to aar- reculminants have been observed in fruiting bodies where myosin function is disrupted in prestalk cells. Culmimants from these transformants form sporeheads but no differentiated stalk, and slug-like structures emerge from within the spore mass (Chen *et al.*, 1998).

The defect in stalk cell induction, like the morphological defect of *aar*⁻, can be rescued by Aar overexpression. Stalk cell induction is rescued to the greatest extent when Aar is overexpressed to a high level although these transformants do not have wild type morphology. As discussed in chapter 4 this may be due to overexpression of Aar inhibiting normal morphogenesis during culmination: this effect is eliminated by monolayer analysis.

Is the monolayer phenotype of *aar*⁻ due to a defect in terminal differentiation of prestalk/prespore cells or an earlier problem with the specification of prestalk/prespore cell types? *aar*⁻ cells express reduced levels of prespore and prestalk B marker genes, which may be sufficient to explain the two-fold decrease in spore/stalk induction compared to wild type. However no increase in *ecmA* expression is observed in the *aar*⁻ mutant so it is not clear what the fate of any potentially "undifferentiated" *aar*⁻ cells is. In addition *aar*⁻ cells transformed with *psA-lacZ*, *ecmA-lacZ* and *ecmB-lacZ* appear to have essentially wild type patterning prior to culmination, as discussed in the next chapter.

The reduction of both prestalk B- and prespore-specific gene expression emphasises the fact that Aar is not regulated in a simple manner by gskA during development. Furthermore, the effects of eliminating or overexpressing Aar in a *gskA*$^-$ mutant background suggest strongly that Aar is not an *in vivo* downstream target of gskA during prestalk:prespore proportioning in the aggregate but that Aar may be able to substitute for the function of an endogenous related molecule at this stage of development.

Chapter 6

The role of Aardvark in developmental axis specification

6.1 Introduction

As described in chapter 4, SEM analysis showed that *aar*⁻ culminants have ectopic tips protruding from the developing spore mass. I termed these ectopic tips "evil twins". Although *Dictyostelium* mutants that have multiple tips at the mound stage have been observed[3], to my knowledge, *aar*⁻ is unique in having more than one tip at culmination. A wild type *Dictyostelium* fruiting body has only one tip and is radially symmetrical about a developmental axis running from tip to base along the stalk tube. Thus *aar*⁻ mutants have developed a new, secondary, developmental axis. Interestingly, duplication of the developmental axis is observed in vertebrates when ß-catenin is misexpressed in the embryo (Fagotto *et al.*, 1996; Funayama *et al.*, 1995; Kelly *et al.*, 1995).

Axis duplication similar to that observed in *aar*⁻ can be induced in *Dictyostelium* by grafting a second slug or preculminant tip onto the prespore region of a wild type preculminant (Rubin and Robertson, 1975). The grafted tip cells appear to organise surrounding cells into a new developmental axis within the spore mass, however the cellular composition of the new axis was not examined in detail (Rubin and Robertson, 1975). Is the *aar*⁻ mutation acting as a "molecular tip graft"? To answer this question I investigated the origin and composition of evil twins.

6.2 *aar*⁻ fruiting bodies have multiple and disrupted stalk tubes

Evil twins arise with high frequency in *aar*⁻ (in 13.6% of culminants examined, see also table 6.1) but almost never in wild type (0.1%). To understand the significance of the evil twins the morphology of *aar*⁻ preculminants was examined in detail. Observation of cryopreserved embedded structures revealed that *aar*⁻ preculminants nearly all contain more than one stalk tube (figure 6.1). Approximately 50% of *aar*⁻ preculminants have stalk tubes that are split symmetrically (figure 6.1a). The remaining preculminants contain secondary stalk tubes, which are not symmetrical with the primary stalk (figure 6.1b). Sectioning of the embedded mutants also reveals that *aar*⁻ stalks are often not continuous along their length (figure 6.1c).

[3] http://glamdring.ucsd.edu/others/dsmith/REMIgenes1998.html

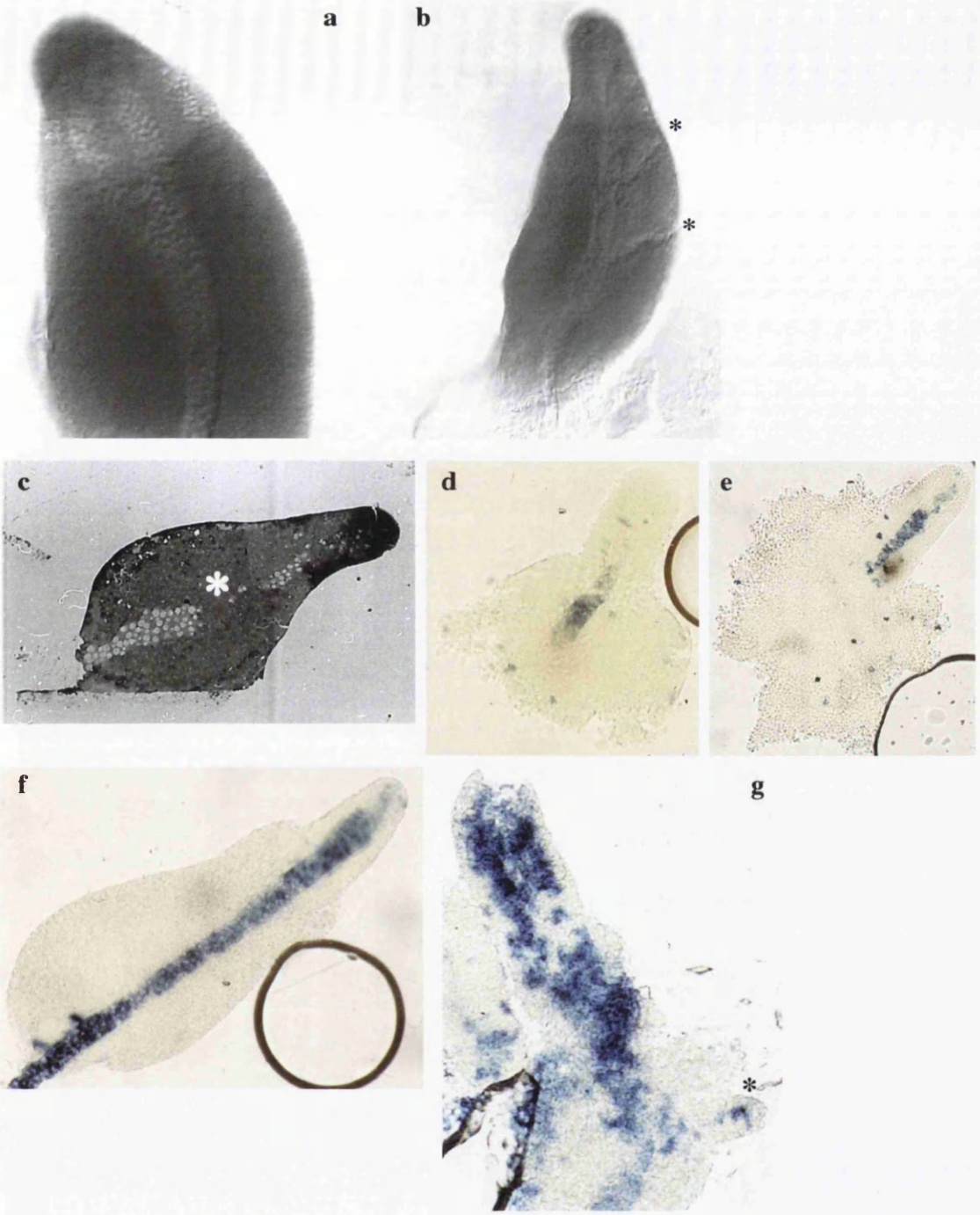


Figure 6.1 *aar*⁻ mutants have disrupted and multiple stalk tubes
a) Early culminant with a symmetrically bifurcated stalk tube in the tip.
b) Whole mount of an early culminant with two ectopic stalk tubes (asterisks).
c) Longitudinal section through a preculminant structure with a non-contiguous stalk tube (asterisk).
d) Wild type Mexican hats show *ST-lacZ* expression exclusively within the developing stalk tube.
e) *aar*⁻ Mexican hats express *ST-lacZ* in the stalk tube, similarly to wild type.
f) Wild type culminants express *ST-lacZ* in the stalk and occasional lower cup cells.
g) *aar*⁻ stalk tubes have a disrupted structure and *aar*⁻ evil twins express *ST-lacZ* within their tip (*).

How does the existence of extra stalks in the *aar*⁻ mutant relate to the formation of evil twins? My hypothesis was that evil twins would contain an ectopic stalk tube, which was the cause of axis duplication. To investigate whether evil twins contained a stalk tube I transformed *aar*⁻ cells with *ST-lacZ*, a stable marker, which in wild type cells is expressed almost exclusively in cells within the stalk tube (Ceccarelli *et al.*, 1991) (figure 6.1d,f). The *ST-lacZ* reporter is driven by the fragment of the *ecmB* promoter which is only derepressed as prestalk cells enter the stalk tube during culmination (Harwood *et al.*, 1993; Harwood *et al.*, 1992b). Before culmination, at the Mexican hat stage, *aar*⁻, like wild type, expresses *ST-lacZ* in the developing primary stalk tube (figure 6.1e). However, as culmination proceeds evil twins are observed which contain *ST-lacZ* expressing cells in a core within their tip (figure 6.1g). This core of *ST-lacZ* expressing cells appears to act as the founder of a new stalk tube: later in development evil twins are observed which contain a complete secondary *ST-lacZ*-expressing stalk tube (refer to figure 6.5f for an example of such a structure). This core of cells in the evil twin is therefore analogous to the core of prestalk AB cells in the wild type slug which form the stalk tube primordium at culmination (Jermyn and Williams, 1991; Sternfeld, 1992).

6.3 Ectopic expression of *ST-lacZ* in *aar*⁻

Analysis of *aar*⁻ mutants transformed with *ST-lacZ* showed that additional cells within the culminant ectopically express *ST-lacZ* (figure 6.2a,b). Ectopic *ST-lacZ* expression is primarily seen in those cells which are in contact with the stalk, particularly near the top of the stalk tube (figure 6.2a,b). These staining cells do not always have a stalk cell morphology but some have the flattened appearance of cells that surround the stalk tube (figure 6.2a). Other staining cells appear to be breaking away from the main stalk (6.2a). However, scattered cells are also seen within the developing spore mass (figure 6.2b), particularly in later culminants, suggesting they may have moved away from the top of the stalk tube. In cross section *aar*⁻ preculminants are seen to contain vacuolated cells, which are assumed to be stalk cells, outside the stalk tube (figure 6.2c). This misplacement of stalk cells could be due to the absence of intercellular junctions at the top of the stalk tube (chapter 4, figure 4.11). The lack of cellulose stalk tube integrity in this region could allow some prestalk or stalk cells to escape into the spore mass rather than moving into the stalk tube. It seems plausible that some of these scattered cells may give rise to the *de novo* stalk tube primordia seen within evil twins.

The entrance to the *aar*⁻ primary stalk tube appears disordered. *ST-lacZ* expression in wild type cells begins below the very tip of the culminant, in the region where the intercellular junctions and stalk tube constriction are observed (figure 6.1f). In the *aar*⁻ mutant *ST-lacZ* expression extends into the cells in the tip, above the region where the constriction should be (figure 6.2a,b). These *ST-lacZ* expressing cells are not vacuolated, angular stalk cells and do not have cellulose walls, as judged by tinopal staining (figure 6.2d,e). Although *ST-lacZ* is usually expressed exclusively in the stalk tube it is not a marker for terminally differentiated stalk cells (Fukuzawa *et al.*, 1997). One possibility is that the presence of *ST-lacZ* expressing cells outside the stalk tube of the *aar*⁻ mutant arises due to their failure to migrate into the stalk tube. To test this, methylene blue staining was used to examine the movement of stalk cells in living culminants. Methylene blue selectively stains the cone of prestalk AB cells in the slug which initiate stalk tube formation (Sternfeld, 1992) (figure 6.2f,g). The inner portion of the basal disc stains blue in *aar*⁻ as in wild type (Sternfeld, 1992) (figure 6.2h,i) indicating normal downward migration of these prestalk AB cells during culmination. Thus prestalk cells can migrate to the base of the culminant and initiate stalk formation, although this does not preclude that the rate of entry of prestalk cells into the stalk may be retarded, particularly later in culmination.

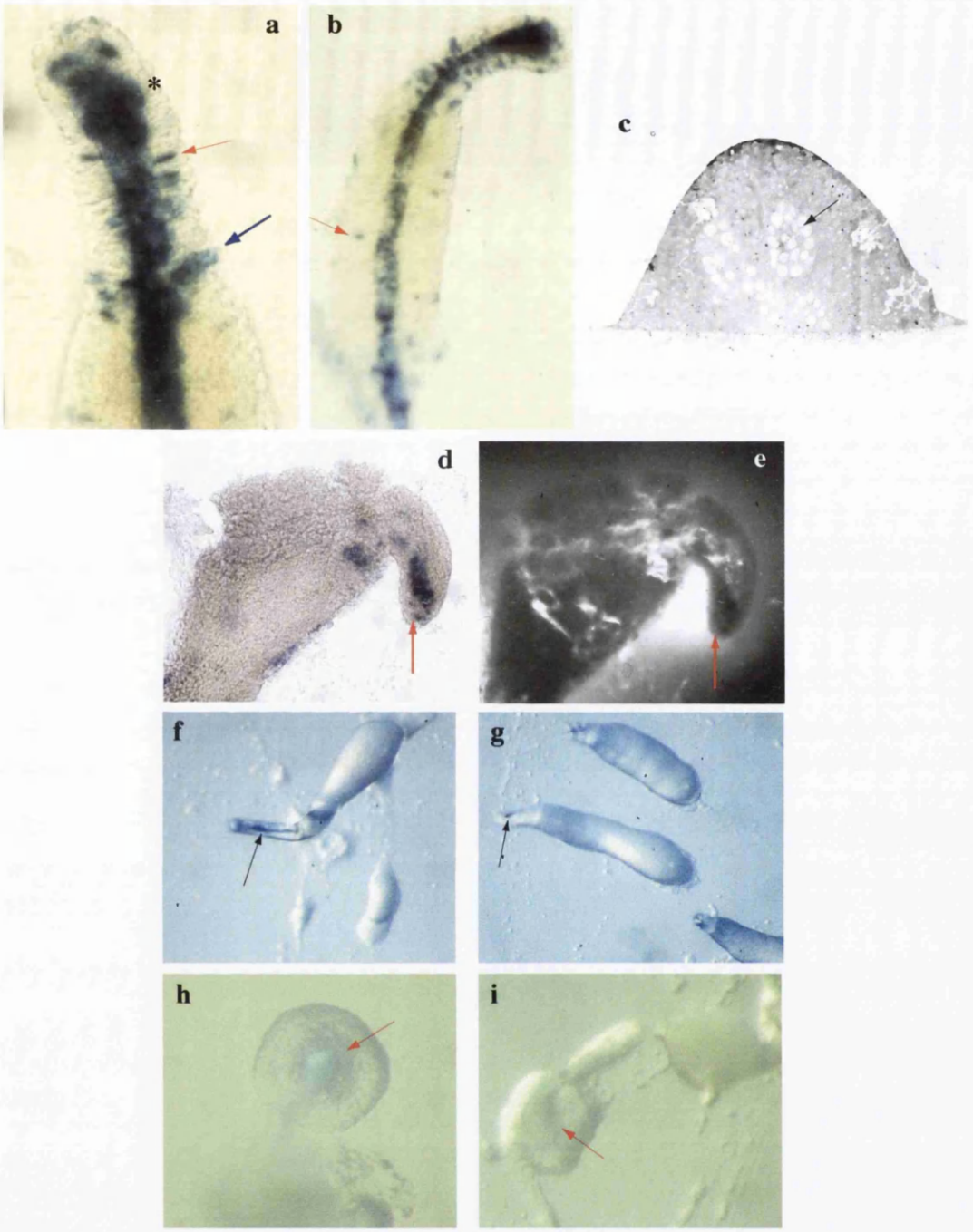


Figure 6.2 *aar*⁻ culminants contain misplaced *ST-lacZ* expressing cells

a) *aar*⁻ culminants have *ST-lacZ* expressing cells outside the stalk tube: in flat prestalk cells (red arrow), in cells which may have broken away from the stalk tube (blue arrow) and in cells above the entrance to the stalk tube (*).

b) *ST-lacZ* expressing cells are also found scattered within the spore mass (arrow).

c) Cross section through an *aar*⁻ culminant showing a narrow stalk tube (arrow) and many scattered vacuolated cells within the developing spore mass.

d), e) The *ST-lacZ* expressing cells above the stalk tube in *aar*⁻ (arrows) are not stalk cells, as they do not stain with tinopal. d) shows *lacZ* expression, e) shows tinopal costaining visualised under UV light

f) Wild type and g) *aar*⁻ cells in the stalk tube primordium of the slug stain with methylene blue (arrows).

h), i) These cells migrate to the centre of the basal disc (arrows) in wild type (h) and *aar*⁻ (i) culminants.

6.4 Evil twins are patterned similarly to wild type structures

Only a small number of cells within the tip of an evil twin express *ST-lacZ*, therefore what is the origin of the remainder of the evil twin? To address this question *aar*⁻ cells were transformed with cell type specific reporter genes: the *ß-galactosidase* gene (*lacZ*) coupled to *ecmA*, *ecmB* or *psA* promoters (Dingermann *et al.*, 1989; Harwood and Drury, 1990; Jermyn and Williams, 1991). These reporters can be used to "fate map" developing structures and are ideal for tracing the origins of cells in *Dictyostelium.* Not only cells that are presently expressing the reporter gene, but also those that have previously expressed the gene, will contain ß-galactosidase activity.

Evil twins show expression of *ecmA-lacZ* restricted to their tip and stalk tube (figure 6.3a,b). This is the same as in wild type culminants where the whole *ecmA* promoter drives *lacZ* expression in the tip and stalk tube (figure 6.3d) (Early *et al.*, 1993; Jermyn and Williams, 1991). *aar*⁻ primary tips and stalks also express *ecmA-lacZ* (figure 6.3a,e). *psA-lacZ* is expressed in the prespore and spore regions of both wild type and mutant culminants and is excluded from the tip (Dingermann *et al.*, 1989) (figure 6.3f,g). The tips of evil twins do not express *psA-lacZ* and are therefore not of prespore origin (figure 6.3c). However cells behind the tip do express *psA-lacZ*, indicating that prespore cells can contribute to the evil twin and give rise to a wild type pattern of cell types within the secondary axis (figure 6.3c).

To trace the origins of evil twins I examined reporter gene expression earlier in development, at the slug stage. Evil twins do not appear to arise from a mispatterning of prestalk or prespore cells in the slug. *aar*⁻ slugs express *ecmA-lacZ* in their anterior one fifth and in scattered anterior-like cells (ALC) within the prespore region, identically to wild type slugs (Gaskell *et al.*, 1992; Jermyn and Williams, 1991) (figure 6.3h,i). *ecmO-lacZ* contains a fragment of the *ecmA* promoter which is activated only in the posterior part of the prestalk A region in the tip and in ALC's (Early *et al.*, 1993) (figure 6.3j). Wild type and *aar*⁻ slugs show virtually identical patterns of *ecmO-lacZ* expression (figure 6.3j,k). Furthermore, wild type and *aar*⁻ slugs also show identical expression of *psA-lacZ* in their posterior four fifths (figure 6.3l,m). Wild type slugs express *ecmB* in the ALC's and in the cone of cells in the tip which form the stalk tube primordium (Jermyn and Williams, 1991) (figure 6.3n). Importantly, *aar*⁻ slugs only contain one cone of *ecmB*-expressing cells in their tip, emphasising that ectopic stalk tube formation is not initiated at the slug stage (figure 6.3o).

Where do the *ecmA*-expressing cells in the tip of evil twins arise from? Since they are not recruited from the prespore cell population, the most likely explanation is that they are anterior-like cells. During culmination the ALC's move out of the prespore region to surround the sporehead, forming the upper and lower cups (Jermyn and Williams, 1991; Sternfeld and David, 1982). If the tip of the slug is removed, ALC's can sort out from the posterior and regenerate a new tip (Sternfeld and David, 1981; Sternfeld and David, 1982). An analogous situation could occur in the *aar*⁻ culminant: the presence of an ectopic "organising signal" could divert ALC's into the tip of an evil twin, which would therefore express *ecmA-lacZ*.

6.5 Ectopic expression of a prestalk B marker in *aar*⁻

Wild type ALC's move in two different directions: some move downwards and constitute the basal disc and lower cup, cradling the bottom of the sporehead, others move upwards to form the upper cup, just below the tip (Jermyn and Williams, 1991; Sternfeld and David, 1982). A subset of ALC's express *ecmB-lacZ* (Gaskell *et al.*, 1992; Jermyn and Williams, 1991), and are a distinct population from those *ecmB*-expressing cells within the stalk tube. Thus wild type *ecmB-lacZ* expression in the culminant shows a characteristic pattern of the upper and lower cups lying perpendicular to the stalk tube (figure 6.4a,d). If *aar*⁻ evil twins are formed from ALC's, this might occur at the expense of upper and lower cup structures.

In *aar*⁻ fruiting bodies a wild type pattern of *ecmB-lacZ* staining is seen at early culmination, with the upper and lower cups and ALC's clearly visible (6.4b,c). However, as development proceeds, cells within the region of the tip outside the stalk tube begin to express *ecmB-lacZ* until, in later culminants, the entire tip is stained blue (figure 6.4e, compare with d). The tips of evil twins also stain throughout with *ecmB-lacZ* (figure 6.4f). This strong *ecmB* expression in the tip does not appear to arise due to the PKA-mediated derepression of stalk-specific *ecmB* outside the stalk tube. Derepression of *ecmB* leads to expression of *ST-lacZ* throughout the tip of the culminant and expression of *ecmB-lacZ* or *ST-lacZ* throughout the tip of the slug (Harwood *et al.*, 1993; Harwood *et al.*, 1992b; Mohanty *et al.*, 1999). The *aar*⁻ mutant does not express *ST-lacZ* throughout the tip of the culminant or misexpress *ecmB-lacZ* in the slug. One possibility is that the ectopic *ecmB* expression arises due to the movement of anterior-like cells into the primary tip and the tip of evil twins late in culmination. However the upper cup and scattered ALC populations do not appear to be depleted at this stage (figure 6.4e). Another possibility is that the *ecmB* expression

in the tips of *aar*⁻ mutants arises *de novo* late in culmination due to an ectopic inductive event. This was not predicted from the expression pattern of *ecmB* on a northern blot (figure 5.2); however the *lacZ* reporter gives no indication of the expression level of the endogenous gene and in addition the number of cells in the tip is relatively small.

6.6 *ST-lacZ* induction in wild type cells mixed with *aar*⁻ cells

In metazoa, axis duplication is due to the cell-autonomous signalling function of Arm/ß-catenin. To address this issue for the *aar*⁻ mutant, wild type and *aar*⁻ cells were mixed and allowed to culminate. When mixed, wild type and *aar*⁻ cells develop with wild type timing and are found evenly distributed throughout developing structures as assayed by neutral red staining of prestalk cells (figure 6.5a,b). A mixture of 50% wild type cells with 50% *aar*⁻ gives rise to *aar*⁻ like culminants, which form evil twins at lower frequency than *aar*⁻ cells alone (table 6.1). If *aar*⁻ cells have a cell-autonomous defect in axis specification then *aar*⁻, but not wild type, cells would be expected to misexpress *ST-lacZ* and contribute to evil twins in mixes. Interestingly, this is not the case. In this situation both wild type and *aar*⁻ cells can contribute to the population of misplaced *ST-lacZ* expressing cells (figure 6.5e,f, table 6.1). When *ST-lacZ* expressing cells are mixed with their cognate untransformed cells they distribute correctly within the fruiting body (figure 6.5c,d).

Even 90% wild type: 10% *aar*⁻ is sufficient to induce ectopic *ST-lacZ* expression in wild type cells and allow wild type cells to form evil twins (figure 6.5g,h) but 1% *aar*⁻ mixed with wild type gives rise to wild type structures (6.5i). The converse mixes (90% or 99% *aar*⁻, 10% or 1% wild type) produce mutant culminants (6.5j,k,l) and misplaced wild type cells are seen (figure 6.5j, table 6.1). This indicates that wild type cells in an *aar*⁻ mutant background behave as *aar*⁻ cells and demonstrates that that the ability to form evil twins is not a cell-autonomous property of *aar*⁻ cells.

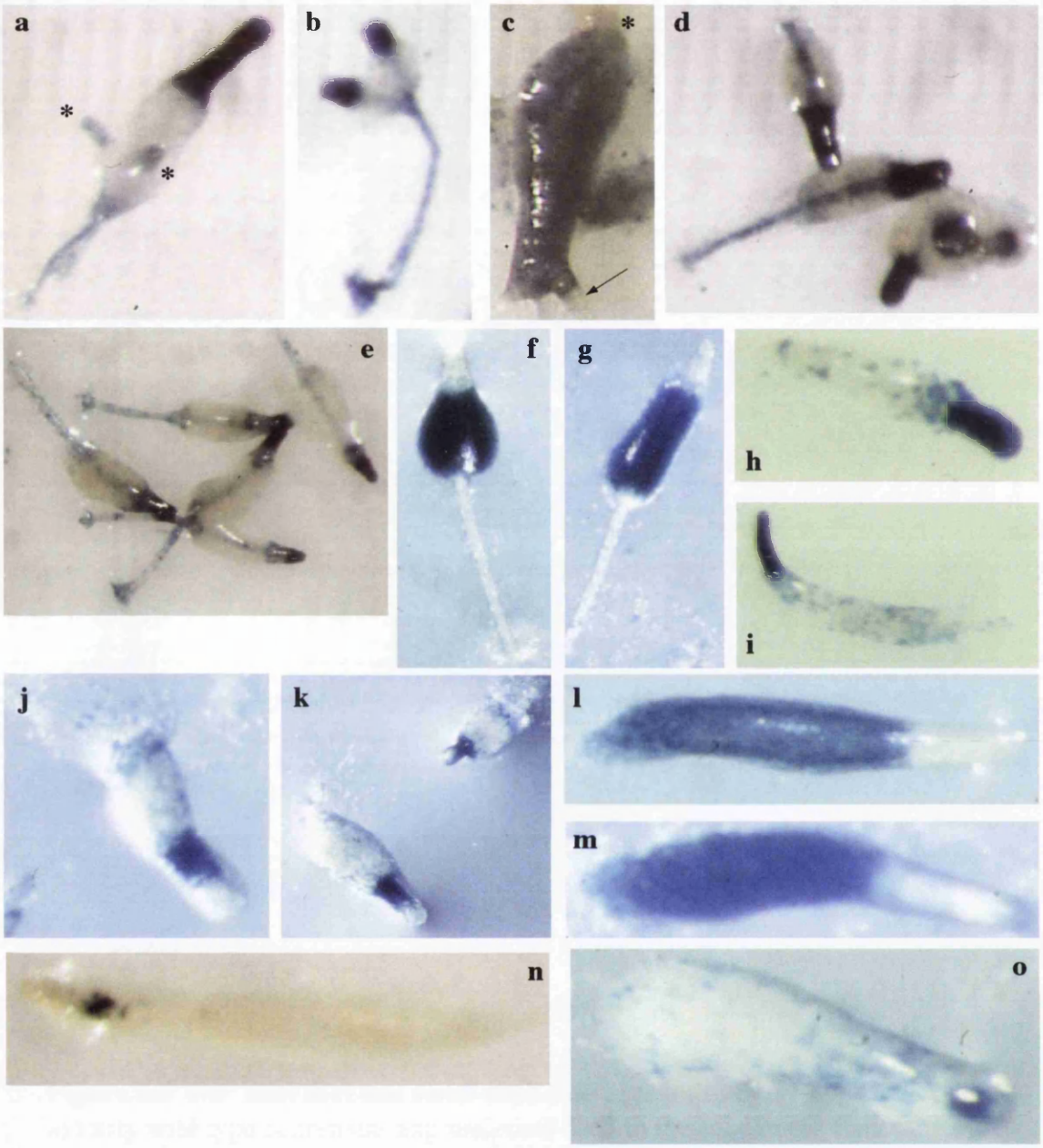


Figure 6.3 Expression of prestalk and prespore markers in wild type and *aar*⁻

a) *aar*⁻ asymmetrical evil twins (*) express *ecmA-lacZ* in their developing tips.

b) *aar*⁻ culminants with symmetrical evil twins express *ecmA-lacZ* indistinguishably in the tip and stalk of the original axis and the tip and stalk of the evil twin.

c) An *aar*⁻ evil twin (arrow) does not express *psA-lacZ* in its tip. The original tip of the fruiting body is marked with an asterisk.

d) Wild type and e) *aar*⁻ fruiting bodies without evil twins show identical *ecmA-lacZ* expression in the tip and stalk tube

f) Wild type and g) *aar*⁻ fruiting bodies without evil twins show identical *psA-lacZ* expression in the developing spore mass.

h) Wild type and i) *aar*⁻ slugs express *ecmA-lacZ* in their anterior one fifth (the tip) and the scattered anterior-like cells in the posterior.

j) Wild type and k) *aar*⁻ slugs both express *ecmO-lacZ* in the posterior half of the tip.

l) Wild type and m) *aar*⁻ slugs express *psA-lacZ* in their posterior four fifths.

n) Wild type and o) *aar*⁻ slugs express *ecmB-lacZ* in a single cone in the tip.

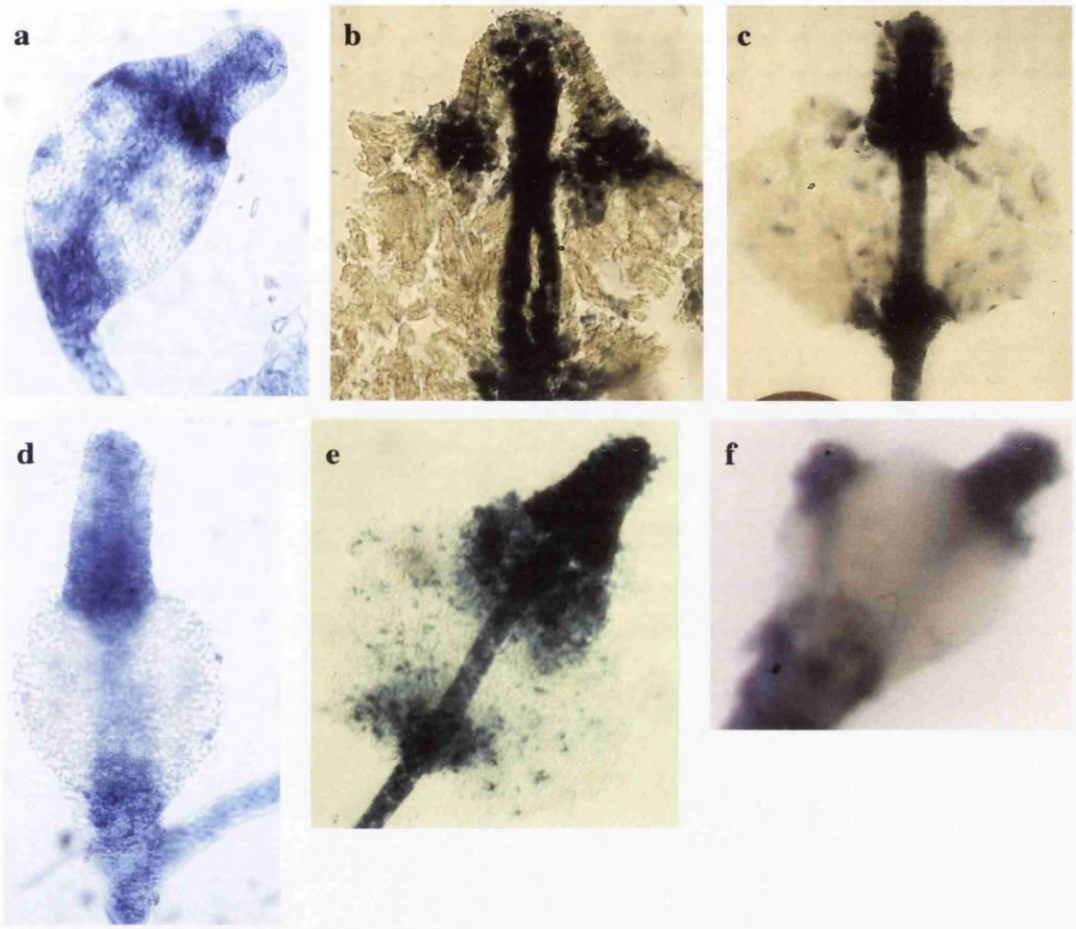


Figure 6.4 *aar*⁻ misexpresses *ecmB-lacZ* late in culmination
a) Early wild type culminants express *ecmB-lacZ* in the cells in the stalk tube, in the upper and lower cups surrounding the spore mass and in scattered anterior-like cells within the spore mass which will later move to the cups (compare with d).
b), c) *aar*⁻ early culminants also express *ecmB-lacZ* in a wild type pattern. Note the double stalk tube in b).
d) Late wild type culminants express *ecmB-lacZ* in the upper and lower cups and the stalk tube. The cells in the tip outside the stalk tube remain essentially free of expression.
e) Late *aar*⁻ culminants express *ecmB-lacZ* in the cups and stalk tube but in addition show strong staining throughout the tip.
f) *aar*⁻ evil twins express *ecmB-lacZ* throughout their tip.

	Wild type (WT)	*aar*⁻	WT 50% *aar*⁻ 50%	WT 90% *aar*⁻ 10%	*aar*⁻ 90% WT 10%	WT 99% *aar*⁻ 1%	*aar*⁻ 99% WT 1%
% evil twins ± sem	0.1 ±0.1	13.6 ±8.5	5.5 ±2.6	2.1	10.0	0.47	12.2
Total fruiting bodies counted	1568	546	853	518	251	425	278
Misplaced *ST-lacZ* cells per culminant ±sem	0* ±0	3.4* ±3.2	1.6 WT ±1.2 1.0 *aar*⁻ ±0.9	1.5+ ±1.0	5.3+ ±6.7		

Table 6.1 The frequency of evil twin occurrence in culminants of wild type (WT), *aar*⁻ and mixes. The mean number of evil twins observed was expressed as a percentage of the total number of upright fruiting bodies counted. Where more than one experiment was performed the standard error of the mean (sem) is shown. The frequency of scattered *ST-lacZ* expressing cells outside the stalk tube (excluding those at the base of the stalk, which are sometimes seen in wild type, and those in the tip above the stalk tube which could not be counted accurately) is also shown. * 50% of wild type or *aar*⁻ cells transformed with *ST-lacZ*. +The cells of the 10% component of the mix were transformed with *ST-lacZ*.

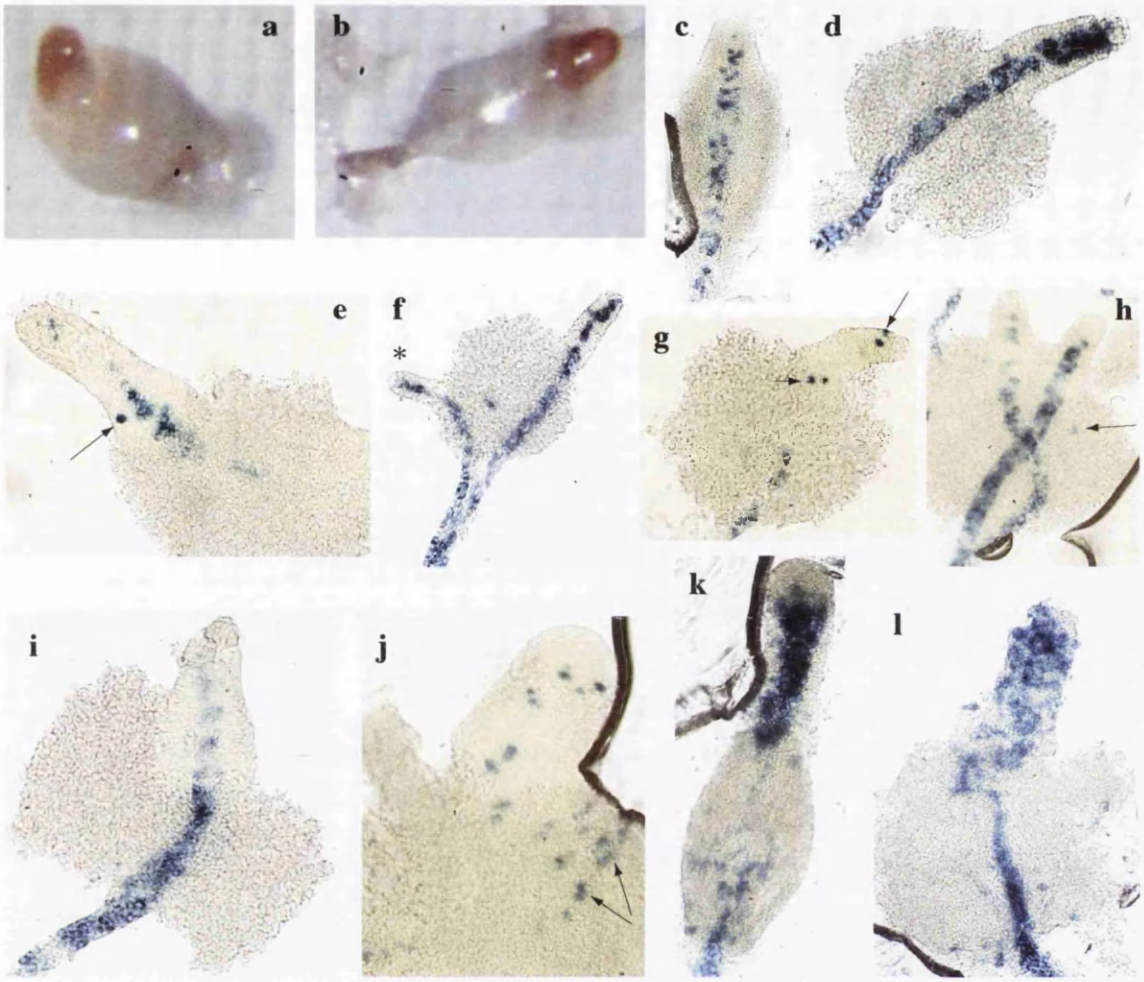


Figure 6.5 Wild type cells express *ST-lacZ* outside the stalk tube in an *aar*⁻ mutant background

a) 50% neutral red-labelled wild type (AX2) cells mixed with 50% unlabelled *aar*⁻ develop identically to the converse mix (b).

c) 50% wild type cells transformed with *ST-lacZ* (AX2-*ST-lacZ*) mix correctly with untransformed wild type cells, as do 50% *aar*⁻-*ST-lacZ* cells with unlabelled *aar*⁻ (d).

e) When AX2-*ST-lacZ* and unlabelled *aar*⁻ cells are mixed 50/50, misplacement of wild type *ST-lacZ* expressing cells is seen (e, arrow).

f) 50% *aar*⁻-*ST-lacZ* cells mixed with unlabelled wild type cells form an *aar*⁻- like structure with an evil twin (*).

g) 10% *aar*⁻-*ST-lacZ* cells in a 90% unlabelled wild-type background are sufficient to cause misplacement of*aar*⁻-*ST-lacZ* cells (arrows).

h) 10% unlabelled *aar*⁻ mixed with 90% AX2-*ST-lacZ* causes misplacement of wild type cells outside the stalk tube (arrow) and duplication of the developmental axis.

i) However, 1% unlabelled *aar*⁻ cells mixed with 99% AX2-*ST-lacZ* form a wild type structure.

j) 10% AX2-*ST-lacZ* cells mixed with 90% *aar*⁻ are able to express *ST-lacZ* outside the stalk tube (arrows).

k) 90% or l) 99% *aar*⁻-*ST-lacZ* cells mixed with unlabelled wild type cells clearly have an *aar*⁻ mutant phenotype.

6.7 Conclusions

The evil twins observed in *aar*⁻ mutants arise from the formation of ectopic tips at culmination, which cause duplication of the developmental axis. The presence of ectopic tips appears to be due to misplaced *ST-lacZ* expressing cells. These misplaced cells could arise by two distinct mechanisms. Firstly, some, but not all (figure 6.2) prestalk cells may fail to enter the stalk tube, due to the disruption of its structure, and hence remain available to form evil twins. If rate of entry of prestalk cells into the stalk tube is limited, a "traffic jam" of *ST-lacZ*-expressing prestalk cells could form. This would explain why cells above the entrance to the stalk tube often express *ST-lacZ* in the *aar*⁻ mutant. Prestalk cells may initiate *ST-lacZ* expression at the correct time, for example by responding to a signal within the tip of the culminant. However some prestalk cells are in the wrong place, being unable to enter into the top of the stalk tube and terminally differentiate. These *ST-lacZ* expressing prestalk cells could move away from the top of the stalk tube and initiate the formation of a new stalk tube and hence developmental axis. Secondly, in the *aar*⁻ mutant ectopic *ST-lacZ* staining prestalk cells appear to be induced within the tip cells outside the stalk tube, presumably by an inductive signal leaking from within the disrupted stalk tube. This is good evidence that a signal from stalk cells must exist.

The correlation between the existence of misplaced *ST-lacZ* expressing cells and the formation of evil twins suggests that these cells have organising ability. These cells appear to initiate the formation of a new stalk tube and signal to surrounding prestalk and prespore cells, generating a secondary developmental axis. An organiser was defined as a group of cells in an embryo which can define a developmental axis and behave autonomously in grafting experiments ((Rubin and Robertson, 1975) and references therein). My data do not prove that the misplaced *ST-lacZ* cells have ectopic organising ability, however it seems very likely. The mixing experiments indicate that there is never a situation where evil twins form but no misplaced *ST-lacZ* expressing cells are present.

Thus the cause of axis duplication in the *aar*⁻ mutant seems to be a specific defect in cell-cell adhesion, namely loss of intercellular junctions in a group of cells at the top of the stalk tube, which disrupts the structure of the culminant and allows misplacement of prestalk cells. Therefore the cause of axis duplication appears to be distinct from the cell-autonomous function of Aar in terminal differentiation (as

outlined in chapter 5), although loss of structure may in turn lead to misplaced inductive signals within the culminant.

Chapter 7

Discussion

The aims of my project were as follows:

- To identify and isolate ß-catenin homologues in *Dictyostelium*
- To investigate whether they are developmentally regulated
- To investigate whether they are associated with the actin cytoskeleton
- To investigate whether they are regulated by gskA

In this chapter I will discuss my findings with reference to each of these aims in turn.

7.1 Are there developmentally regulated ß-catenin homologues in *Dictyostelium*?

I have shown that at least three distinct proteins related to ß-catenin/Armadillo and its close relative plakoglobin are present in developing *Dictyostelium* cells. Ddplako is a 69kDa protein immunologically related to vertebrate plakoglobin and is expressed first when cells begin to stream and form cell-cell contacts; its expression persists at a similar level until the end of development. The Ddarm protein is immunologically related to vertebrate ß-catenin and *Drosophila* Armadillo (Arm) but not to plakoglobin. A 90kDa form of Ddarm is present in vegetative cells and throughout development: its expression increases greatly in late development, in slugs and culminants. The upregulation of 90kDa Ddarm is concomitant with the appearance of a 130kDa form of Ddarm. Whether 90kDa and 130kDa Ddarm are two proteins, or a single protein which undergoes a modification late in development, is unknown. Only cloning of the genes for Ddarm and Ddplako will show whether their amino acid sequences are related to ß-catenin/Armadillo and plakoglobin.

The *aardvark* gene is expressed late in development, the message can be detected in total mRNA from the slug stage onwards. *aar* encodes a protein of 697 amino acids with ten Arm repeats, which are related to the repeats of Arm/ß-catenin/plakoglobin, *S. cerevisiae* Vac8p and uncharacterised human and plant genes. Aardvark repeats are degenerate and although they show only ~20% identity to Arm/ß-catenin/plakoglobin repeats, functional homologues of ß-catenin have been cloned in *C. elegans* which show a similar degree of identity to their vertebrate counterparts (Eisenmann *et al.*, 1998). In addition, the amino terminus of Aar harbours potential gsk3ß/gskA phosphorylation sites and a region with similarity to the α-catenin binding site of Arm/ß-catenin/plakoglobin. Thus Aar is a good candidate for a functional homologue of Arm/ß-catenin/plakoglobin in *Dictyostelium* development. In support of this, *aar*⁻ mutants have defects at the culmination stage of development. *aar*⁻ culminants

lack intercellular junctions, can form more than one developmental axis and have a cell-autonomous defect in terminal stalk and spore differentiation in monolayer culture. As ß-catenin/Armadillo function in cell-cell adhesion, axis specification and cell-autonomous fate specification this provides further support for Aar being a true homologue of these metazoan proteins. This is the first time that Arm/ß-catenin/plakoglobin-related proteins have been found in a non-metazoan multicellular organism and suggest that the control of multicellular development by these proteins arose very early in evolution. However the mechanism by which Aar is involved in axis formation appears to be different to that seen in animal systems, as discussed in more detail below.

7.2 Are *Dictyostelium* ß-catenin-like proteins associated with the actin cytoskeleton?

7.2.1 Aar mediates junctional formation

Aar is required for the formation of junctions around the stalk tube during culmination: reintroduction of Aar into the *aar*⁻ mutant restores the formation of these junctions. Overexpression of Aar leads to an excess of disorganised and misplaced junctional structures. This suggests that the presence of a tightly regulated pool of "junctional" Aar may be the rate-limiting factor in junction formation *in vivo*. Similar observations have been made in other systems: elimination of *arm* from the *Drosophila* ovary reduces intercellular adhesion and eliminates a subset of junctions which contain actin and Arm (Peifer *et al.*, 1993). A moderate *arm* allele disrupts the formation of the *Drosophila* embryonic epithelium (Cox *et al.*, 1996), eliminating cadherin-containing junctions, cell adhesion and cell polarity in a very similar manner to *aar* in *Dictyostelium*. In mammalian cells stable cadherin-based adhesion could be generated in the absence of ß-catenin when α-catenin was fused directly to the tail of E-cadherin, but no dynamic regulation of the adhesion complex could occur, suggesting ß-catenin acts as a regulatable linker in the formation of adherens junctions (Nagafuchi *et al.*, 1994). Likewise an the increase in intercellular adhesion observed in mammalian cells expressing Wnt-1 may be due to an increase in ß-catenin regulating the formation of N-cadherin adhesive complexes (Hinck *et al.*, 1994c). Although maternal depletion of ß-catenin in the *Xenopus* oocyte did not affect intercellular adhesion (Heasman *et al.*, 1994), loss of plakoglobin led to embryos with adhesion defects (Kofron *et al.*, 1997). ß-catenin-null mice also have adhesion defects (Haegel *et al.*, 1995). Junctional

organisation in plakoglobin null mice is disrupted but not eliminated: hybrid junctions containing ß-catenin are seen in place of discrete organised adherens junctions and desmosomes (Ruiz *et al.*, 1996).

Ideally Aar should be reintroduced into the *aar*⁻ mutant under the control of its own (as yet uncloned) promoter. Perhaps a later developmental promoter, such as the *ecmA* promoter, would allow expression of Aar in a more suitable location in the culminant and provide better phenotypic rescue. However since Aar also plays a role in spore cell differentiation in monolayers, full rescue might still not be achieved. *In situ* hybridisation can be used in future do determine the location of *aar* expressing cells and allow a more suitable choice of promoter.

An important unresolved issue is whether Aar localises to the intercellular junctions in the culminant and directly regulates junctional formation. Given its protein sequence, this seems highly likely. My generation of an anti-Aar antiserum should allow the intercellular localisation of Aar to be determined in the near future. An altenative strategy would be to design a DNA construct which, when transformed into cells, would integrate into the *aar* locus and introduce an epitope tag onto one end of the endogenous *aar* gene. Introduction of green fluorescent protein (GFP) onto Aar would allow the behaviour of the protein to be followed in living structures throughout development.

Interestingly, the antiserum to human plakoglobin, which recognises Ddplako on western blots, localises to the intercellular junctions regulated by Aar. Both ß-catenin and plakoglobin are found metazoan adherens junctions, although their binding to a single cadherin molecule is mutually exclusive ((Aberle *et al.*, 1994; Hulsken *et al.*, 1994). The association of plakoglobin with adherens junctions is weaker than that of ß-catenin and plakoglobin cannot substitute for ß-catenin in the initial formation of adherens junctions (Aberle *et al.*, 1994; Haegel *et al.*, 1995; Ozawa *et al.*, 1989). Ddplako cannot substitute for the loss of Aar in the culminant, therefore Aar and Ddplako could have a similar relationship to vertebrate ß-catenin and plakoglobin, respectively, in junctional formation. Alternatively, it is possible that the anti-plakoglobin antiserum could cross-react with junctional Aar in TEM sections, although this is not the case on western blots.

The intercellular junctions organised by Aar at the top of the stalk tube hold together a ring of actin, the tension in which compresses the stalk tube in this area (this work; M. J. Grimson, unpublished data). A contractile bundle of actin filaments linked to the plasma membrane has also been described in the unicellular protostelid

Planoprotostelium aurantium (Spiegel *et al.*, 1979). The filaments constrict the basal region of the cell as it elongates to form a stalk tube and may also organise the sites of cellulose stalk tube synthesis to the membrane in this region (Spiegel *et al.*, 1979). Thus an actin-based mechanism for building a stalk tube may be conserved in all slime mould species. Similar actin rings held together by cadherin/catenin-containing junctions exist in metazoan systems and appear to fulfil similar tension-generating roles. Actin rings are assembled in wounded mammalian epithelia, which require E-cadherin mediated cell-cell contacts and possibly actin-myosin contractile interactions to generate the forces necessary for wound closure (Danjo and Gipson, 1998). A similar purse-string closure of cells occurs in the *C.elegans* hypodermis to generate a long, thin worm. This process requires cadherin, α-catenin and ß-catenin homologues (Costa *et al.*, 1998). In the developing *Drosophila* intestine, a ring of punctate junctional structures containing Arm is seen in the endothelial cell layer surrounding the gut lumen (Peifer, 1993), this structure appears very similar to that seen in the layer of cells surrounding the top of the stalk tube in *Dictyostelium.* In this respect the junction-containing prestalk cells resemble an endothelium: the cells are highly polar in morphology and the junctions and actin ring reside close to the apicolateral surface of the cells, juxtaposing the stalk tube. In addition these cells are likely to secrete the matrix proteins ecmA and ecmB from their apical surface into the stalk tube (M. J. Grimson, pers. commun.). The junction-containing cells define a new subtype of prestalk cells and indicate that even a simple metazoan such as *Dictyostelium* requires highly specialised cell types to regulate the complex morphogenetic movements occurring during culmination. Whether the junctions contain a molecule similar to classical cadherins is currently unknown. The only *Dictyostelium* protein with homology to cadherins, DdCAD-1 (Wong *et al.*, 1996) has no cytoplasmic tail for catenin binding and functions only during early development. The existence of sequences with homology to α-catenin/vinculin in the *Dictyostelium* developmental cDNA database (Morio *et al.*, 1998) strongly supports the notion of a true cadherin/catenin system being present in the junctions surrounding the stalk tube.

7.2.2 The role of Aar in axis formation

The formation of evil twins by the *aar*⁻ mutant appears to be a consequence of the disrupted stalk tube structure. Mixing up to 90% wild type with *aar*⁻ cells does not rescue the stalk tube disruption or axis duplication phenotype. The presence of a few *aar*⁻ cells seems sufficient to disrupt the structure of the actin ring and stalk tube: *aar*⁻

or wild type cells in a 90% wild type background can become misplaced. However the actin ring stretches around 20-30 cells (M. J. Grimson pers. commun. and figure 4.11b) so if one cell in this region (less than 10% of the cells) was *aar*⁻ and could not form junctions the whole structure might fall apart like a broken elastic band. Indeed mixing 1% *aar*⁻ cells into a wild type background leads to wild type structures suggesting that the presence of a very small percentage of *aar*⁻ cells is insufficient to disrupt the wild type stalk tube structure. In addition, 1% *aar*⁻*-ST-lacZ* cells in a wild type background behave as wild type cells (A. J. Harwood pers. commun.).

As a consequence of the lack of intercellular junctions in the *aar*⁻ mutant, the cellulose stalk tube normally secreted by the stalk cells within it is severely disrupted. The data presented in chapter 6 suggest that this disruption of structure leads to ability of *aar*⁻ cells (or wild type cells in an *aar*⁻ background) to form fruiting bodies with more than one axis. The disrupted stalk tube appears to allow cells on the outside to express *ST-lacZ* and this correlates with the formation of evil twins. Ectopic *ST-lacZ* expression may be due to *ST-lacZ* expressing cells failing to enter the stalk tube, instead becoming misplaced within the spore mass. Alternatively or additionally an inductive signal may escape from within the stalk tube and induce evil twin formation. Interestingly, low molecular weight factors with a possible role in terminal stalk cell differentiation, STIF and SDFs, have been identified. STIF acts synergistically with DIF to induce stalk cell differentiation and *ST-lacZ* expression (Yamada *et al.*, 1997). SDFs induce spore and stalk cell maturation in monolayers (Anjard *et al.*, 1998a). Whether STIF or SDFs are associated with the stalk tube *in vivo* is unknown.

The best way to look for leakage of an inductive signal from the stalk tube would be to examine stalk-specific gene expression and cell movement in a living culminant. Ideally, *aar*⁻ culminants expressing the *ecmB* stalk-specific (*ST*) promoter fragment driving green fluorescent protein (GFP) expression could be examined in real time. Would cells expressing *ST-GFP* always originate in the stalk and then move to other places within the culminant, or could *ST-GFP* cells outside the stalk induce *de novo* expression? This experiment could also confirm that misplaced *ST*-expressing cells initiate the formation of evil twins: thus although potentially difficult to perform it is an important experiment to be attempted in the future.

Many experiments have been performed to elucidate whether the role of ß-catenin/Arm in metazoan axis specification is mediated via cell adhesion or by an independent signalling function. Axis duplication by abolition of Aar most likely arises due to a disruption of the structure of the culminant. Thus at a first glance the

mechanism for loss of Aar initiating an ectopic axis appears quite different from that whereby ß-catenin induces axis duplication. Overexpression of ß-catenin in *Xenopus* or Zebrafish duplicates the embryonic axis (Fagotto *et al.*, 1996; Funayama *et al.*, 1995; Kelly *et al.*, 1995). The level of ß-catenin protein present in these experiments is very high and axis duplication most likely arises due to saturation of the endogenous ß-catenin degradation machinery leading to ectopic nuclear translocation of ß-catenin and dorsal cell fate specification. Depletion of maternal ß-catenin from the *Xenopus* oocyte abolishes dorsal axis specification without affecting cell-cell adhesion, as a substantial amount of membrane-associated ß-catenin was still present in these experiments (Heasman *et al.*, 1994; Kofron *et al.*, 1997). In the *Drosophila* embryo, Wg signal modulates levels of cytosolic Arm without grossly affecting intercellular adhesion or levels of junctionally associated Arm (Peifer *et al.*, 1994b; Peifer *et al.*, 1994c; Sanson *et al.*, 1996).

However these experiments do not completely rule out the possibility that changing Arm levels via Wg signalling may be subtly affecting adhesion. For example, *zw3* mutant embryos have reduced tyrosine phosphorylated Arm (Peifer *et al.*, 1994b) and tyrosine phosphorylation of ß-catenin correlates with decreased junctional stability in mammalian cells (Hinck *et al.*, 1994b). Reduction of Arm function in discrete clones of cells (analogous to my mixing experiments) is thought leads to axis duplications via the incorrect juxtaposition of cell types in *Drosophila* leg and wing imaginal discs. *arm* clones in ventral cells of the leg or in distal cells of the wing are respecified as dorsal or proximal, respectively, due to their lack of Arm (Peifer *et al.*, 1991). Although this respecification mirrors that seen in *wg* and *dsh* mutants (Klingensmith *et al.*, 1994; Theisen *et al.*, 1994) it is not clear whether the *arm* cells are respecified due to changes in gene expression or cell adhesion. Importantly, in imaginal discs there is very little correlation between *wg* expression and Arm accumulation (Peifer *et al.*, 1991). In leg and wing imaginal discs endogenous Arm is largely membrane-associated and has a polar distribution within each cell (Peifer *et al.*, 1991), therefore reduction of *arm* function could lead to changes in junctional structure. The juxtaposition of ventral/distal and misplaced dorsal/proximal cells is proposed to lead to cell-cell interactions which generate an ectopic axis (Peifer *et al.*, 1991). The loss of Aar transforms "junctional" prestalk cells into "non-junctional". The outcome of this is misplaced inductive signals, as in *Drosophila*, leading to duplication of a developmental axis.

7.2.3 Misplaced *ST-lacZ* expressing cells may act as organisers

Previous experiments have shown that the tip of *Dictyostelium* is an organiser: isolated tips can reform entire fruiting bodies (Sternfeld and David, 1981) and first finger, slug or preculminant tips the grafted onto the outside of another fruiting body can induce secondary axes (Rubin and Robertson, 1975). The range of evil twin morphologies observed in *aar*⁻ mutant culminants parallels that observed previously, where tip grafts initially induced small bifurcations within the spore mass and eventually gave rise to an entire new axis with its own stalk (Rubin and Robertson, 1975). Interestingly, control experiments in which no tip was grafted but the structure of the fruiting body was perturbed could induce ectopic axis formation at low frequency (Rubin and Robertson, 1975). This could be analogous to a part of the mechanism which is operating in the *aar*⁻ mutant.

The grafting experiments were performed with whole tips, i.e. a group of heterogeneous cells. My data suggest that single cells may have the ability to act as organisers and recruit the cells around them to form new axes. This has not been shown in other systems: classical grafting experiments always involve groups of cells and because growth occurs concomitant with development in metazoa a single injected or mutant cell within an embryo will always divide. However, the frequency of ectopic *ST-lacZ*-expressing cells is higher than the frequency of ectopic axes (Table 6.1 and figures 6.4 and 6.5). Perhaps a new axis is more likely to form in the presence of a group of misplaced *ST-lacZ* cells which would have more potent organising ability.

In the *aar*⁻ mutant, a signal from the misplaced *ST-lacZ* cells may induce surrounding cells to switch on *ecmB*, both in the primary tip and in the tips of evil twins. Introducing a small number of wild type *ecmB-lacZ* expressing cells into an *aar*⁻ background and vice versa would allow this possibility to be tested. I predict that wild type cells located in the tips of evil twins would express *ecmB-lacZ* and that 1% *aar*⁻ cells in a wild type structure would not express *ecmB-lacZ* ectopically.

7.3 Are *Dictyostelium* ß-catenin homologues regulated by gskA?

The expression patterns of Ddplako and Ddarm in wild type and *gskA*⁻ cells suggest that either protein could be regulated by gskA. Expression of Ddplako is reduced in the *gskA*⁻ mutant relative to wild type and Ddplako is specifically depleted from a membrane-associated pool in *gskA*⁻ cells. This demonstrates that Ddplako is downregulated in the absence of gskA activity. Thus if Ddplako is regulated directly by gskA, the mechanism for this is different to the destabilisation of Armadillo/ß-catenin

homologues by gsk3ß in metazoa. The expression pattern of Ddarm suggests that it could, in part, be regulated by gskA in a manner analogous to the metazoan model. In wild type cells levels of Ddarm are lowest at the mound stage, when gskA activity is high (Plyte *et al.*, 1999). Conversely, when gskA activity is likely to be reduced, during culmination (Plyte *et al.*, 1999), Ddarm levels are elevated, suggesting it is stable in the absence of gskA activity. In *gskA*⁻ cells Ddarm levels are elevated relative to wild type in the mound. However the reduced expression of Ddarm during culmination in the *gskA*⁻ mutant suggests that Ddarm must also be regulated by other mechanisms.

The Aar sequence contains several potential sites for gskA phosphorylation, suggesting that it could be regulated by gskA during development. The observed *gskA*⁻ mutant phenotype is caused by the elimination of gskA activity at the mound stage, resulting in an increased formation of prestalk cells and a decrease in the prespore cell population. Stalk cell induction by DIF in monolayer cultures is not repressed by cAMP in *gskA*⁻ cells. I found no evidence for the *gskA*⁻ phenotype being caused by misregulation of Aar. The *aar*⁻ mutant phenotype becomes apparent later in development than the *gskA* phenotype and *aar*⁻ cells show reduced prestalk and prespore differentiation. In addition, stalk cell induction by DIF in monolayer cultures is repressed by cAMP in *aar*⁻ cells, as in wild type cells. However, two caveats exist when attempting to define a genetic interaction between *aar* and *gskA* by examining the loss-of-function mutants.

Firstly, gskA may have developmental functions after the mound stage in wild type cells which are not apparent from the *gskA*⁻ mutant phenotype. To ascertain whether Aar could be regulated by gskA after the mound stage will require a demonstration that Aar can be phosphorylated by gskA. This can be done simply *in vitro* (Ryves *et al.*, 1998), perhaps using synthetic peptides encoding the regions of Aar containing putative gskA phosphorylation sites. The phosphorylation status of Aar could be examined *in vivo* during wild type and *gskA*⁻ development, or in cells treated with lithium or serine/threonine phosphatase inhibitors, by assessing the mobility of Aar on SDS-PAGE. It is interesting to speculate that while Aar is undetectable in wild type cells with my antiserum, it could be stabilised in *gskA*⁻ cells. In addition detection of Aar by immunofluorescence in developing wild type or *gskA*⁻ structures will provide information about the developmental regulation of its intracellular location. A genetic test of whether and how Aar is regulated by gskA phosphorylation *in vivo* would be to express forms of Aardvark lacking some or all of the putative gskA phosphorylation sites in wild type and *gskA*⁻ cells. This form of Aar, by analogy with ß-

catenin/Armadillo, might escape the normal regulatory degradation mechanisms. Thus Aar lacking gskA phosphorylation sites may have a more profound effect on wild type development than overexpressed full length Aar, but have the same effect as full length Aar when transformed into *gskA*$^-$. Alternatively, if gskA phosphorylation of Aar leads to its stabilisation then a form of Aar lacking the gskA sites might be unstable in wild type cells and not cause an overexpression phenotype.

The second caveat of studying the loss of function mutants is that Aar may be redundant with other proteins during development. Overexpression of Aar from the beginning of development in the *gskA* mutant rescues its morphology. This rescue occurs, at least in part, due to a reduction in the prestalk B cell population in the mound. One interpretation of this result is that exogenously expressed Aar is substituting for the loss of a related protein, such as Ddplako, which is usually upregulated by gskA activity in the aggregate. One prediction of this is that a *Ddplako*$^-$ mutant would have a phenotype similar to *gskA*$^-$. Another interpretation is that a ß-catenin-like molecule, such as Ddarm, is normally stabilised in the *gskA*$^-$ mutant. Overexpressed Aar may compete for a binding partner of Ddarm and target Ddarm for degradation, as is seen when plakoglobin is moderately overexpressed in mammalian cells (Salomon *et al.*, 1997), but Aar may be unable to fulfil the cellular function of Ddarm. Thus Aar would be acting in a dominant negative fashion to rescue the *gskA*$^-$ morphological defect.

7.4 Summary

I have demonstrated the existence of three developmentally regulated ß-catenin/Armadillo/plakoglobin-related proteins in a non-metazoan eukaryote, *Dictyostelium discoideum.* Two of these proteins, Ddarm and Ddplako, have altered expression in a *gskA*$^-$ mutant and could potentially be regulated by gskA. gskA is the *Dictyostelium* homologue of gsk3ß/zw3, a protein known to regulate ß-catenin/Armadillo during pattern formation in metazoa. The third protein, Aardvark (Aar), has not yet been demonstrated to be directly regulated by gskA. However Aar contains several consensus sites for gskA phosphorylation in its amino terminus, similar to those seen in its metazoan relatives.

Ddplako and Aar are likely to be associated with components of the first intercellular junctions discovered in *Dictyostelium.* These junctions link to the actin cytoskeleton and are similar to metazoan adherens junctions. A large percentage of Ddplako is found in a membrane/cytoskeletal pool in cell extracts and an antiserum which recognises Ddplako localises to the intercellular junctions. Culminants lacking

Aar do not form junctions and as a consequence, the structure of the stalk tube is disrupted. This allows some cells which should enter the stalk during culmination to become misplaced within the fruiting body and organise the formation of a secondary developmental axis. Aardvark is also required cell autonomously for the efficient terminal differentiation of both stalk and spore cells.

Metazoan ß-catenin and Armadillo are also required for the formation of cell-cell junctions, axis specification and developmental signalling. The mechanism by which Aar is involved in axis formation, via regulation of specific cell-cell contacts, appears to be different from that seen in animal systems. Whether, like their animal counterparts, *Dictyostelium* Armadillo proteins are able to directly regulate gene expression remains to be discovered. This work shows that Armadillo/ß-catenin-like proteins exist in a multicellular non-metazoan and demonstrates that the dual role of these proteins in intercellular adhesion and developmental signalling arose before the evolution of the metazoa.

Chapter 8

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