

# Chapter 2

## ***Dictyostelium* Development: A Prototypic Wnt Pathway?**

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### **Abstract**

Although Wnt signaling is ubiquitous within the animal phylogenetic group, it is unclear how it evolved. Genes related to the components of Wnt pathway are found in other eukaryotes and one of the most studied of these non-metazoan organisms is the social amoeba *Dictyostelium discoideum*. This organism contains the enzyme GSK-3 and a  $\beta$ -catenin homolog, Aardvark (Aar). Both are required to regulate pattern formation during multi-cellular stages of *Dictyostelium* development. Aar is also required for formation of adherens junctions, as seen in animals. Finally, analysis of the completed *Dictyostelium* genome shows there to be 16 Frizzled (Fz) gene homologs. This chapter discusses *Dictyostelium* development and the role of these proteins.

**Key words:** *Dictyostelium*, GSK-3,  $\beta$ -catenin, Aar, Frizzleds, pattern formation.

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### **1. Introduction**

Wnt signaling is universally found with the metazoan group, but does it exist outside the animals? Results over the last 20 years indicate that elements of the canonical Wnt pathway, involving GSK-3 and  $\beta$ -catenin, exist in a number of major non-metazoan groups. This raises questions about its evolutionary origins and in particular the relationship between the role of  $\beta$ -catenin in cell signaling and the cytoskeleton. One of the best studied non-metazoan Wnt-like pathways is found in the social amoeba, *Dictyostelium discoideum*. This chapter describes the unique biology of the Dictyostelid group and how it can be used to investigate an evolutionarily distinct, perhaps prototypic, signal pathway related to the Wnt pathway of animals.

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## 2. *Dictyostelium* Biology and Development

*Dictyostelium* belong to the taxonomic group termed amoebozoa. These are mainly soil amoeba that feed via phagocytosis of particulate matter, bacteria in the case of the Dictyostelids. For many years, based mainly on erroneous molecular phylogenetic data using ssRNA sequences, *Dictyostelium* was considered as a fundamentally different group from other eukaryotes. However, recent evidence produced by Baldauf and Doolittle (1) points to a much closer relationship to animals. Through their pioneering work, it is now clear that animals, fungi, and the amoebozoa, belong to a common arm of the eukaryotic evolutionary tree. This much closer relationship to animals is reflected in the genetic composition apparent from the whole genome sequence (2).

The information from the complete genomic sequence is freely accessible through the curated database, known as dictyBase (3). The associated Web pages contain a Blast server for searching both genomic DNA sequences and the complete collection of predicted 12,000 open reading frames (ORFs) and proteins. The database also contains a complete *Dictyostelium* research paper bibliography linked to each gene page. This is the ideal entry point for new researchers entering this field, or those from other fields wishing to make comparative phylogenetics of a protein family of interest.

Utilizing the genetic resources available for *Dictyostelium* is relatively straight forward, as it has a compact, haploid genome with few introns and homologous recombination occurs at high frequency. This means that almost any gene can be isolated by PCR and ablated by targeted gene disruption. The haploid genome makes *Dictyostelium* ideal for insertional mutagenesis, which is most commonly carried out using the plasmid-based REMI method (4). The genetic source material has been further expanded by the generation of a gene bank of more than 6,000 non-redundant sequenced cDNA clones (5). As large numbers of cells can easily be grown from protein extraction, *Dictyostelium* is readily amenable to biochemical analysis. Finally, *Dictyostelium* cells are excellent for molecular cell biological analysis; for example, there are a wide range of GFP and epitope tag vectors for marking proteins and their microscopical analysis. This multidisciplinary approach makes *Dictyostelium* a powerful model system to investigate complex cell signaling processes.

By far the most significant feature of *Dictyostelium* is its unusual survival strategy triggered in response to nutrient starvation. Here the up to  $10^5$  cells develop into a differentiated and cell patterned multicellular structure, known as a fruiting body (6). This is triggered by a combination of loss of nutrients and high cell density, and can be readily induced in the laboratory by washing cells free of nutrients and then plating them or culturing

them in the absence of further nutrients. Cells are either grown in association with bacteria, normally *Klebsiella pneumoniae* or B strains of *Escherichia coli*, or liquid medium. All cell strains can be grown with bacteria; however, only cells that carry a set of three “axenic” mutations, such as the AX2 and AX3 parental strains, can grow in medium (7). Multicellular development can be carried out either on non-nutrient agar; moist nitrocellulose filters or to a limited extent in shaking suspension in buffer. Importantly, growth arrests at the beginning of development offering the opportunity to investigate phenotypic effects without influence of altered cell growth and division.

*Dictyostelium* development is synchronous and takes 24 hours, except for some variability in the length of the slug stage for cells developed on non-nutrient agar. This basic synchronicity makes it relatively easy to obtain large quantities of cells at the same developmental stage. As *Dictyostelium* cells begin development from unicellular amoebae, the first 8 to 10 hours of development consists of cells coming together through a process of chemotaxis toward pulses of extracellular cAMP. During this aggregation stage, cells are essentially homogeneous. There are however small variations in cell behavior due to differences in nutritional state and position in the cell cycle when starvation begins, which can influence the ultimate cell fate (8, 9). Cells suppress growth phase genes and induce aggregation specific genes during this early phase of development; these latter genes upregulate the cellular machinery to mediate extracellular cAMP signaling and promote cell adhesion (10, 11). As cells can be induced to form terminally differentiated cell types when plated in low density monolayer cultures, these differences in cell history or cell adhesion are not essential for cell development (12, 13), although they could influence morphogenesis during multicellular development.

Multicellular development (**Fig. 2.1**) begins as soon as the cells have formed an aggregate, with cells expressing cell specific genes that mark their ultimate fate in the terminal differentiated structure, the fruiting body. This structure consists of a large spherical head containing 80% of the original cells after they have differentiated into spores. The spore head is supported by a stalk, comprising of dead stalk cells; the remaining 20% of the cell population. Although the most notable feature of the fruiting body is the long, slender stalk of 4 to 5 mm in length, there are other ancillary structures that help support the stalk and spore head. The spore head is cradled between cells that form cup like structures on its top and bottom (14). The stalk is attached to the substratum by the basal disc. The cells within this structure are morphologically the same as those of the stalk itself; however they arise from a different population within the aggregate (15).

Within the newly formed aggregate, cells differentiate into three precursor populations (16). The majority form prespore

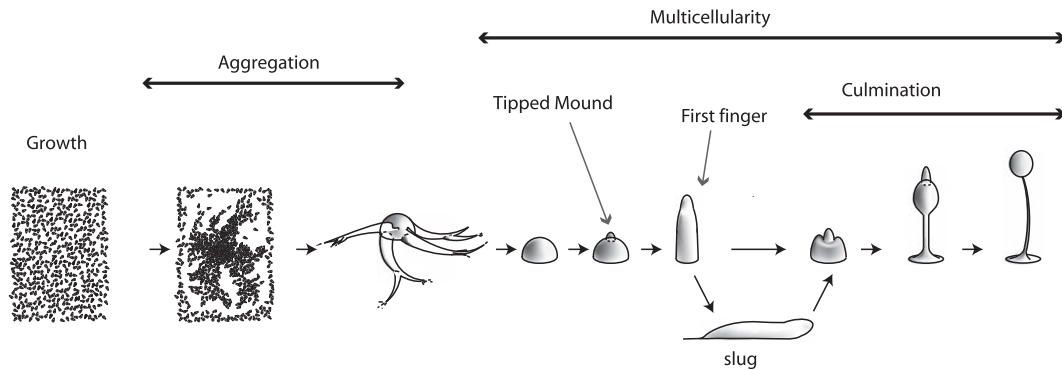


Fig. 2.1. Development of *Dictyostelium*.

cells. A particularly useful marker for looking at GSK-3 control of this process is the gene *pspA*. When linked to a *lacZ* marker gene (17), it can be seen that this gene is expressed in cells as soon as they enter the aggregate. This gene, as with other prespore specific genes, is induced by extracellular cAMP (18). The cells that ultimately form the stalk, express the marker *ecmA* and although this is a secreted matrix protein, its promoter can be used to mark the population of prestalk cells. These are known as pstA cells. The aggregate also contains pstB cells; these express the related gene, *ecmB*, and form the basal disc cells. Stalk cell differentiation and expression of these marker genes requires DIF-1, a dichlorinated phenyl hexanone (19, 20). Although this is a potent stalk cell inducer in isolated cells, genetic evidence has revealed that there are other regulatory factors that control the pattern and timing of stalk and basal disc formation in the multicellular structure (21).

Prestalk cells appear to form at random within the aggregate, but quickly sort so that pstA cells fill the tip that forms onto the top of the aggregate (the tipped mound stage), whereas the pstB cells move to the basal region. The tip region has the properties of an embryonic organizer throughout the remaining multicellular development. The tip region of the mound extends so that a long finger structure is formed (first finger stage). This may topple over and form a motile slug structure. This is phototactic and is thought to move the developing structure to locations that will aid spore dispersal.

When migration is finished, developing structures enter the culmination stage. Within the tip region, pstA cells begin to differentiate into stalk cells. They do this in a very defined manner and always in the center of the tip and with newly differentiated cells joining the elongating stalk at the top. As pstA cells join the stalk they begin to express *ecmB*, and hence are known as pstAB

cells. The growing stalk penetrates through the prespore region to embed in the basal disc. As the stalk continues to grow, it lifts the prespore cell mass off the substrate. Late during this preculmination stage, as the stalk reaches its full length, the prespore cells differentiate into mature spores (22). Spores can survive for prolonged periods of time without nutrients, but when nutrients are restored, either following dispersal or by plating in the laboratory, they germinate and re-establish the growing population.

Both *gskA* and *aar* mutants have distinctive effects on the terminally differentiated *Dictyostelium* fruiting body, which makes them ideal for morphologically based genetic screens. As described previously, once obtained, mutants can be easily investigated either within multicellular structures by use of marker genes (*see Chapter 3 in Volume 2*), or by controlling the inductive conditions in isolated cell cultures (*see Chapter 4 in Volume 2*). The phenotypes of *gskA* and *aar* mutants reveal further developmental complexity and are discussed in the following sections.

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### 3. GSK-3

*Dictyostelium* contains a GSK-3 ortholog, *gskA*, which has very similar properties to the vertebrate GSK-3 $\beta$  enzyme (23, 24). There is no detectable GSK-3 activity in a *gskA* null mutant indicating that there is only one GSK-3 gene in *Dictyostelium*. Analysis of the *Dictyostelium* kinome (25) identified a related protein kinase gene, *glkA*, which has an intermediate sequence homology between cdk and GSK-3 kinases. The functions and substrates of this kinase are currently unknown. *glkA* particularly differs from *gskA* in lacking the Axin binding site present in all eukaryotic GSK-3 proteins. Exchange of this binding site for the equivalent region from *glkA* has dramatic effects on GskA function in *Dictyostelium* leading to severe effects on the motility of aggregating cells and altered regulation of gene expression (26).

As seen in animals, GskA is a multifunctional kinase that is involved in cell processes in growing cells and throughout *Dictyostelium* development. GskA is not essential for cell growth, but *gskA* null mutants are compromised in cell growth (27). It is required for regulated gene expression at the transition between growth and development, but with no phenotypic effects on the entry into development (28). However, mutant cells do appear to have a slight chemotaxis effect, and aggregate slightly faster than wild-type cells. The reason for this is unknown. GskA phosphorylates the *Dictyostelium* StatA protein to control the rate of nuclear export (29), a regulatory mechanism similar to that seen for NF- $\kappa$ B. These regulatory functions are very reminiscent

of the GSK-3 mediated signaling in animals, but are unlikely to function within a Wnt-related pathway.

The fruiting bodies of *gskA* null have a distinctive phenotype with a small spore head and an enlarged basal disc (27). This phenotype is most pronounced when cells are grown in association with bacterial, and is relatively weak in cells grown in axenic liquid medium. The cause of this difference is unknown. This morphology suggests a defect in pattern formation, and this can be first observed in the aggregate at the mound stage, where the proportion of *pstB* cells expands at the cost of the prespore cells. Interestingly, by use of the strain differences and the weaker phenotype of cells grown in liquid culture, a second cell patterning defect can be seen in *gskA* null mutants during later developmental stages as *ecmB* is expressed throughout the prestalk zone (30). This suggests that not only does *gskA* control the proportion of prespore to *pstB* cells, it acts to repress expression of the *pstB* marker, *ecmB*, in all other prestalk cells.

Extracellular cAMP is a common factor between these events. cAMP is required for prespore and spore induction but represses stalk cell induction and expression of *ecmB* (31). By mediating the effects of cAMP, *gskA* is able to control the induction of all of these cell populations. Consistent with this, GskA activity increases as cells enter the aggregate, and cAMP activates GskA activity in aggregation competent cells (32). Loss of the cAMP receptor *cAR3* prevents the cAMP stimulated increase of GskA activity, although it should be noted that it does not decrease the basal level of activity. Furthermore *cAR3* null mutants have a similar cell patterning defect and exhibit a loss of cAMP repression of *ecmB* gene expression and stalk formation to *gskA* null mutants (32).

Biochemically downstream of *cAR3* is an unusual protein kinase ZakA that contains both a functional tyrosine kinase and serine kinase domain (33). The tyrosine kinase domain is able to directly phosphorylate GskA on the equivalent of the Tyr-216 amino acid in GSK-3 $\beta$ . Loss of ZakA causes the same phenotype as seen in *gskA* and *cAR3* null mutants. There is a paralog of Zak1 in the *Dictyostelium* genome, known as Dpyk4 (25). Loss of ZakA does not prevent all tyrosine phosphorylation of GskA, only that stimulated by cAMP (unpublished observation). It is possible that this second kinase is responsible for the residual GskA phosphorylation, but this has not yet been tested. Interestingly, loss of another cAMP receptor *cAR4*, has the opposite phenotype to loss of *gskA*. These mutants have elevated expression of prespore genes, reduced prestalk gene expression and stalk cell formation is hypersensitive to cAMP (34, 35). Biochemically, *cAR4* stimulation elevates tyrosine dephosphorylation of GskA, counteracting the effect of ZakA (36). Given that *cAR4* has a lower affinity for cAMP than *cAR3* does, this may establish a threshold response in which *gskA* is inactive at low and high levels of cAMP, but active at intermediate concentrations.

#### 4. Aardvark: A $\beta$ -catenin-related Protein

*Dictyostelium* contains a  $\beta$ -catenin-like molecule, known as Aardvark (*aar*). Although it does not arrest development, loss of Aar causes a reduction in the expression of the prespore-specific gene *pspA* during multicellular development (37). The effect of loss of *aar* on cells developed in shaking culture is more striking as cAMP is no longer able to induce *pspA* expression. In monolayer, culture spore-cell formation in the *aar* mutant is reduced, although not completely lost. When Aar is overexpressed, the opposite result is seen as cAMP hyper-induces *pspA* expression. This hyper-induction requires GskA activity, linking it to the GskA-mediated events described in Section 3. However, loss of *aar* does not lead to increased *ecmB* expression or loss of cAMP expression, restricting the effects of Aar to the prespore induction pathway.

These results show that the GskA-Aar pathway operates differently from the canonical Wnt pathway of animals, where Wnt negatively regulates GSK-3 through protein-protein interactions suppressing the repressive effects of GSK-3 phosphorylation on  $\beta$ -catenin. In *Dictyostelium*, GskA activity is controlled by phosphorylation and acts positively on Aar to induce *pspA* expression (Fig. 2.2). The *Dictyostelium* pathway in some respects resembles the non-canonical Wnt pathway that is mediated by mom-2 during early nematode development. Here, genetic evidence indicates that the Wnt protein mom-2 via a Frizzled, mom-5, acts positively on GSK-3 to induce endoderm specification. GSK-3 appears to mediate its effects via a positive interaction with a  $\beta$ -catenin ortholog WRM-1 (38). WRM-1 achieves this by de-repression of the negative effects of a TCF/LEF-related protein, POP-1, via a serine/threonine protein kinase, LIT-1 (39). The mechanism by which Aar regulates transcription is not yet clear.

#### 5. Adherens Junctions

Unexpectedly, given the loose packed nature of the *Dictyostelium* multicellular aggregate, it was discovered that some cells form strong cell-cell junctions that appear very similar to the adherens junctions of animal epithelia (37). These *Dictyostelium* junctions form during the culmination stage and are only present in around 1000 cells situated in a collar-like structure close to the top of the stalk tube. Immuno-EM with antiserum raised against Aar protein localizes to the cell junctions. The junctions were originally missed due to their lability in chemical fixation; however they are persevered after rapid cryopresevation and freeze substitution and can easily be visualized by EM. They can also be directly seen at



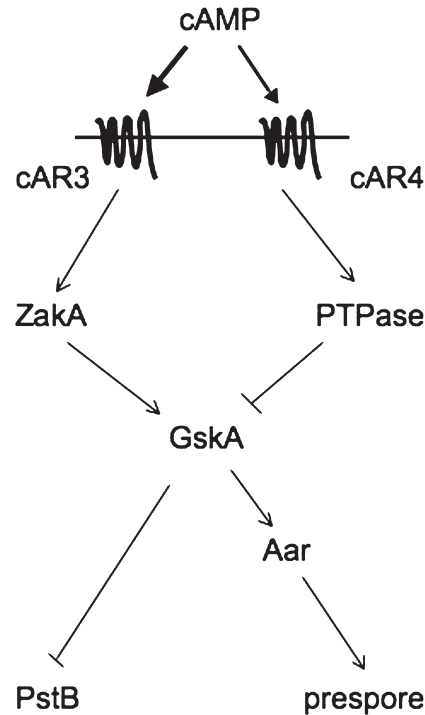


Fig. 2.2. Schematic diagram to summarize GskA-Aar signaling pathway.

a lower resolution in living cells by use of GFP fused F-actin binding proteins (37) or GFP-Aar (unpublished results).

Disruption of the *aar* gene causes a complete loss of these cell junctions and Aar overexpression increases their number and size. These observations demonstrate that Aar expression is both necessary and limiting for junction formation. Interestingly loss of *aar* does not block fruiting body formation. Aar mutants however have weak stalks causing the fruiting body to frequently collapse, suggesting that the adherens junctions are required for the mechanical integrity of the stalk tube. The matrix that forms the stalk tube, the extracellular material that surrounds the mature stalk cells, is a complex laminate with individual fibres laid down by both stalk cells and those prestalk cells surrounding the tube. The function of the *Dictyostelium* adherens junctions may be to polarize secretion of matrix components to the cell surface adjacent to the stalk tube (Fig. 2.3). To date, however, no direct visualization of vesicle transport to this surface has been possible.

Loss of stalk tube integrity can also explain an unusual phenotype where the *aar*-null mutant forms one or more additional stalks (40). These arise during the culmination stage and branch from the main stalk tube. As they develop, they reorganize



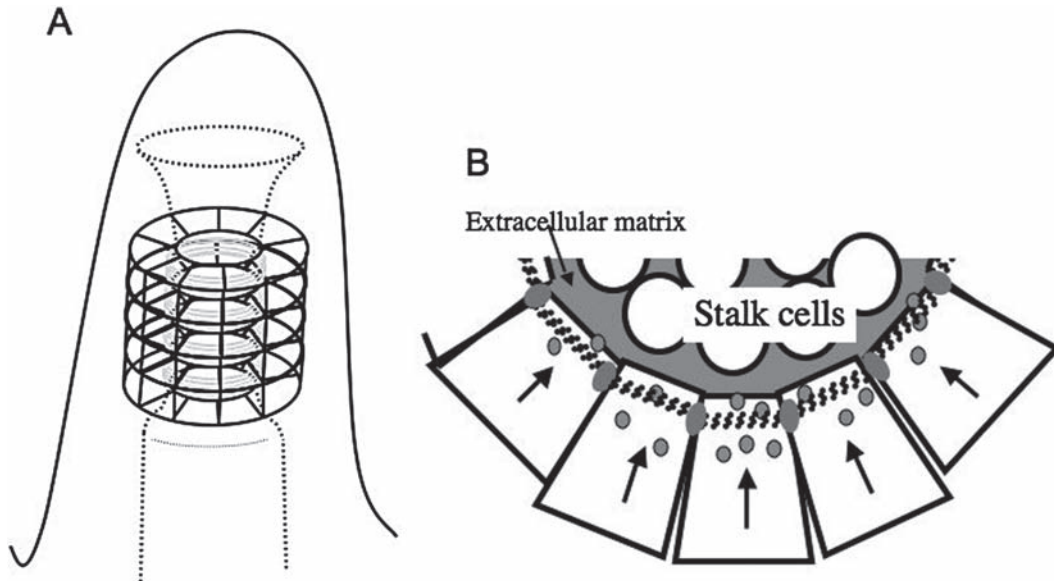


Fig. 2.3. *Dictyostelium* adherens junctions. (A) Cells connected by adherens junctions form a collar toward the top of the stalk tube. The junctions of each cell are connected via actin filaments, effectively forming a ring of F-actin that constricts the stalk tube. (B) In planar view, the presence of the junctions forms apical/basolateral polarization. This allows vectorial transport of vesicles toward the matrix surrounding the stalk cells.

the cells around them and form additional spore heads. At the cellular level, the new stalks form due to the ectopic induction of pstAB cells on the outside of the stalk tube. Mixing mutant and wild-type cells demonstrates that super-nummery stalk formation does not arise through axis duplication, but is a cell non-autonomous event due to misplaced signaling. We believe that they arise due to leakage of a stalk inducing signal through damaged sections of the stalk tube.

## 7. Other Components of Wnt Signaling Pathways

Are there other components of the Wnt signaling pathway in *Dictyostelium* genome? With one exception, there seems to be no other close protein orthologs specific to the animal Wnt pathways; in fact, the Wnt proteins themselves and downstream components such as Dsh, Axin, and APC proteins have not been found. There are, however, generic components, such as a CKI protein kinase, and proteins containing Wnt-related domains, such as the DEP domain of *Dictyostelium* ORF DDB0205035. One of the problems with data mining for animal components in *Dictyostelium* is the very high A-T content of the genome. This

genome bias is seen at the protein level were there is genetic drift to amino acid sequences encoded by codons that have an A-T bias. Coupled to the large degree of degeneracy of the Wnt signal pathway components seen between animal orthologs, may mean that searching the *Dictyostelium* genome will meet only limited success.

The exception is the Frizzled (Fz) proteins. An initial search of the genome carried out by Prabhu and Eichinger in 2006 suggested that there could be as many as 25 Frizzled-like receptors in the *Dictyostelium* encoded in the genome (41). All genes have homology through their trans-membrane domain, however, only 16 contain putative cysteine-rich domain (CRD) present in the animal Fz protein family. These have been denoted as Frizzled/Smoothed-like (*fsl*) genes. Of these *fsl* genes two, *fslJ* and *fslK*, contain, in addition to the CRD domain, a KTXXXW sequence motif, which is required for activation of the canonical pathway in animals. The functions of these genes are not yet known.

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## 8. Conclusions

*Dictyostelium* has many of the features associated with animal signaling systems and contains a number of key components of the canonical Wnt pathway. The study of these pathways in *Dictyostelium* is still in its infancy. To date, we have established that there are both similarities and differences between *Dictyostelium* and animal signal pathways. As *Dictyostelium* offers many advantages as a model system to study cell signaling, further study of the function, interactions and cell biology of GskA and Aar is likely to throw up new insights regarding how components of the Wnt signaling pathway operate in a non-metazoan context.

A key observation is that the Aar protein possesses both signaling and cytoskeletal functions, as seen in animals, whereas the signaling function is different from the canonical pathway, its association with adherens junctions appears conserved. This moves the question of which came first to more evolutionary distant groups than the amoebozoia.  $\beta$ -catenin-related proteins exist in other eukaryotic groups, and may be involved in signaling in organisms in which adherens junctions are not present (42). For example, adherens junctions are not found in plants, but in *Arabidopsis* the  $\beta$ -catenin-related proteins Arabidilo-1 and -2 possess a signaling function in the control of lateral root hair development (43).

**Chapters 3 and 4 in Volume 2** provide protocols for two of the key techniques used to investigate the functions of GskA and Aar. There is no doubt that they will prove useful for further

study of this signaling system. For more general techniques, see the *Dictyostelium discoideum Protocols* volume in the Methods in Molecular Biology series (44).

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Wnt Signaling

Volume 2, Pathway Models

Vincan, E. (Ed.)

2009, XVI, 494 p., Hardcover

ISBN: 978-1-60327-468-5

A product of Humana Press