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Apicomplexan plastids as drug targets

Geoffrey I. McFadden and David S. Roos

Prokaryotic metabolic pathways in the relict plastid of apicomplexan parasites make this organelle a promising target for drug development. The parasiticidal activity of several herbicides and antibacterial antibiotics is suspected to be a result of their ability to inhibit key plastid activities.

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Chloroplasts are chlorophyll-containing organelles found in plants and algae. These organelles come in many colors, including green, red and brown, as well as non-photosynthetic colorless forms. Technically, it is more accurate to describe the whole group as plastids, reserving the term chloroplasts for green plastids. Although photosynthesis is generally the defining function of plastids, these organelles also carry out other metabolic activities that make them indispensable, even to non-photosynthetic plant and algal cells.

Plastids of all colors originate from endosymbiotic cyanobacteria and retain a degree of autonomy. For example, all known plastids contain, within the confines of at least two bounding membranes, a DNA genome and transcription and translation systems. During an estimated 500 million years of intracellular survival within their eukaryotic hosts, they have undergone substantial modification. However, plastid systems remain fundamentally bacterial in nature, making them very different from the cytoplasmic, eukaryotic systems of their hosts.

The recent identification of plastids in parasites of the phylum Apicomplexa, including *Plasmodium* (the causative agent of malaria), *Toxoplasma*, *Eimeria*, *Babesia*, *Theileria*, *Sarcocystis* and *Hepatozoon*[1–4], has opened a Pandora's box of fascinating questions for parasitologists and plastid biologists alike. What is the evolutionary origin of the apicomplexan plastid? What is its function? What opportunities does this newly recognized compartment with its bacterial-like systems offer for combating the important diseases caused by these parasites? This review examines these questions, focusing specifically on the plastid as a target for parasiticidal drug design.

Origin of the apicoplast

All plastids, including the apicomplexan plastid (or 'apicoplast'), arose by endosymbiosis of a cyanobacterial-like prokaryotic cell[5]. Engulfment of this cell, referred to as the primary endosymbiotic event, generated a plastid characterized by two membranes, such as those found in red and green algae, plants and glaucophytes. Following this primary endosymbiosis, plastids are thought to have been transferred laterally into several eukaryotic lineages that normally lacked plastids, by a process known as secondary endosymbiosis. In secondary endosymbiosis, plastid-containing primary endosymbionts were themselves engulfed and retained by other eukaryotes. A wide variety of secondary endosymbionts, exhibiting a range of reduction in the complexity of the engulfed eukaryote, is known to protistologists. In many cases, virtually all the cell

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contents, except the plastid, have been lost[5]. The only tell-tale sign of secondary endosymbiosis in some organisms is the presence of extra membranes around the plastid; secondary plastids have four (or sometimes three) bounding membranes[5]. Despite some conflicting initial reports[2,3], it is now clear that the *Toxoplasma gondii* and *Plasmodium falciparum* apicoplasts are, in fact, surrounded by four membranes (Fig. 1). The presence of four membranes, and the distinctive mechanism of targeting nuclear-encoded proteins to the apicoplast[6–8], are both indicative of a secondary endosymbiotic origin.

Identifying the alga that was the source of the apicomplexan endosymbiont is more difficult. Algae are classified on the basis of pigment content but the apicoplast appears to lack photosynthetic pigments (however, see Ref. 9). The organization and sequence of the apicoplast genome paints a somewhat confusing picture. Phylogenetic trees based on elongation factor Tu (encoded by *tufA*) and other genes suggest a green-algal ancestry[3]; however, inference based on the highly diverged sequences of apicomplexan genes is difficult, limiting confidence in the resulting phylogenies[10]. In contrast to this molecular-systematic analysis, apicoplast genome organization (including the relative location of the ribosomal-protein genes) is more indicative of a red algal endosymbiont[10–12], leaving the identity question unresolved.

The apicoplast as a drug target

DNA replication

Plastid genomes are similar to those of their bacterial progenitors in that they are supercoiled DNA circles[13]. At 27–35 kb, the apicoplast genome is the smallest known plastid genome[1], but it appears to have retained a circular, supercoiled architecture. Early electron micrographs of the genome indicated circularity (reviewed in Ref. 12), and the antimalarial activity of the fluoroquinolones (inhibitors of bacterial type II topoisomerases) hinted at the presence of a DNA gyrase[14]. Further proof that a plastid-specific DNA gyrase has a role in apicoplast genome replication has been provided by Weissig *et al.*[15], who have shown that the fluoroquinolone ciprofloxacin only affects the 35-kb plastid, whereas an etoposide, VP-16, an inhibitor of eukaryotic topoisomerase II, affects both the 35-kb plastid and the nuclear chromosomes of *P. falciparum*. These data suggest the existence of both eukaryotic and prokaryotic type II topoisomerases in the malaria parasite. A candidate for the eukaryotic nuclear-encoded topoisomerase has been suggested[16] but the apicoplast gyrase remains uncharacterized. The apicoplast genome does not encode a gyrase[1], suggesting that the enzyme is nuclear-encoded and targeted into the plastid. Fichera and Roos have demonstrated that ciprofloxacin kills *T. gondii* by blocking apicoplast-DNA replication, thus proving the plastid is an effective drug target[17]. Ciprofloxacin is also moderately active against *Cryptosporidium parvum*[18], another apicomplexan parasite that might possess an apicoplast[19].

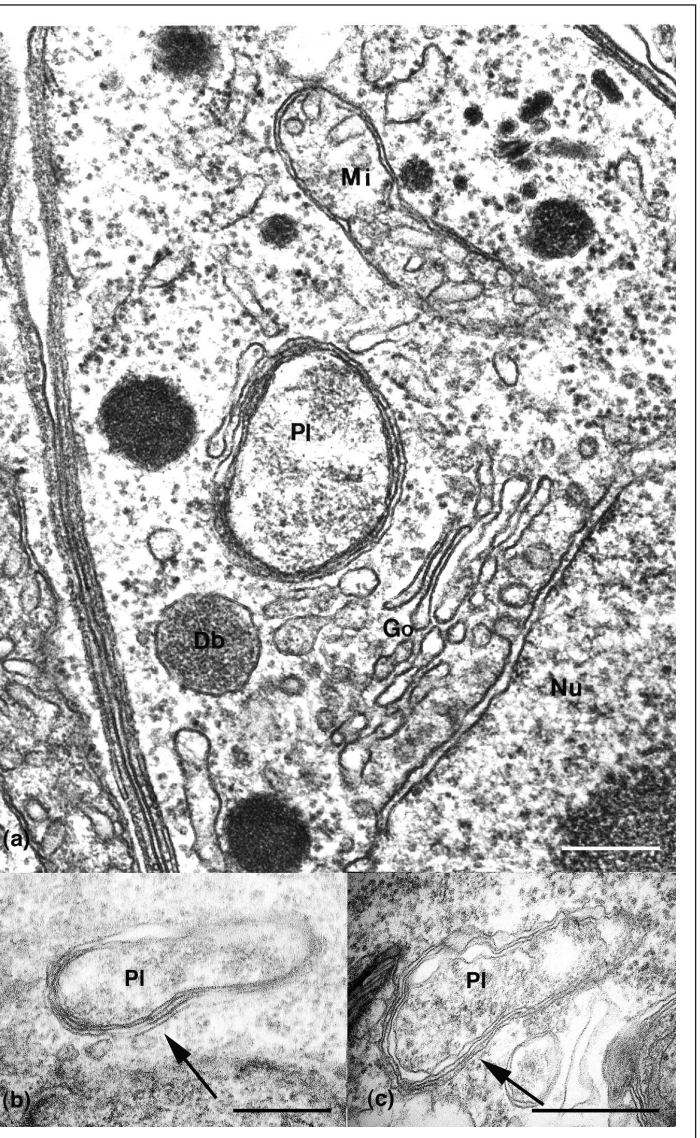

Fig. 1. (a) Transmission electron micrograph of *Toxoplasma gondii* showing the apicoplast (Pl) surrounded by four membranes. The 35-kb plastid genome has been localized to this compartment by *in situ* hybridization[2,3]. **(b)** High magnification of a dividing plastid from *T. gondii* showing a dumbbell shape and four bounding membranes (indicated by an arrow). **(c)** High magnification of the plastid from a *Plasmodium falciparum* gametocyte with four bounding membranes (indicated by an arrow). Abbreviations: Db, dense body; Go, Golgi apparatus; Nu, nucleus; Mi, mitochondrion. Scale bars = 400 nm.

Transcription and RNA processing

Plastid transcription utilizes an RNA polymerase that is homologous to that of cyanobacteria and other eubacteria: the so-called α_2, β, β', DNA-dependent RNA polymerase[20]. This polymerase recognizes promoters with consensus -10 and -35 sequences via the σ-factor and transcribes polycistronic RNAs from plastid DNA operons. The apicoplast genome encodes the β and β' subunits of the polymerase (encoded by the *rpoB*, *rpoC1* and *rpoC2* genes), strongly suggesting that it uses a similar transcription system[1]. Sigma factors, such as that encoded by *rpoD*, are nuclear-encoded in plants and algae[21] and the absence of this

Table 1. Drugs and herbicides proposed to target plastid activities in apicomplexan parasites[a]

Metabolic activity	Drug/herbicide	Putative target	IC_{50}	Remarks	Refs
DNA replication	Ciprofloxacin	Plastid DNA type II topoisomerase (*gyrA*)	*Pf* 50 μM[b] *Tg* 30 μM *Cp* 250 μM	Confirmed by direct observation in *Tg*	14,15,17, 18
RNA transcription	Rifampicin	Plastid RNA polymerase β-subunit (*rpoB*)	*Pf* 3 μM *Tg* 3 μM[b]		22,44
Protein translation	Clindamycin	Plastid 23S rRNA (*rrnL*)	*Pf* 20 nM[b] *Tg* 10 nM[b] *Cp* 20 μM	Strong indirect evidence for activity in plasmid	17,18,24, 28
	Erythromycin		*Cp* ~40 μM		18
	Azithromycin		*Pf* 2 μM *Tg* 2 μM *Cp* 90 μM		18,24,27, 28
	Spiramycin		*Tg* 40 ng ml^{-1}		24,28
	Thiostrepton		*Pf* 2 μM *Tg* NA	Strong indirect evidence for activity in plastid	30,32,33
	Micrococcin		*Pf* 35 nM	Strong indirect evidence for activity in plastid	31
	Chloramphenicol		*Pf* 10 μM[b] *Tg* 5 μM[b] *Cp* 300 μM		18,24,27, 34
	Doxycycline	Plastid 16S rRNA (*rrnS*)	*Pf* 100 μM *Cp* 200 μM	Might also target mitochondrial protein synthesis	18,34
	Tetracycline		*Pf* 10 μM *Tg* 20 μM *Cp* 100 μM	Might also target mitochondrial protein synthesis	18,27,34
Photosynthesis	Toltrazuril	D1-water-splitter protein of photosystem II (*psbA*)	*Et* 5 μM	Putative presence of target in Apicomplexa requires substantiation	9
Amino acid biosynthesis	Glyphosate	5-enopyruvyl shikimate 3-phosphate synthase (*aroA*)	*Pf* 3 mM *Tg* 2 mM *Cp* 6 mM	Pathway probably cytosolic rather than plastid	40,41
Fatty acid biosynthesis	Thiolactomycin	β-ketoacyl-ACP synthase III (*fabH*)	*Pf* 50 μM *Tg* 100 μM[b]	No direct evidence for mechanism of parasiticidal activity	6

[a]Abbreviations: ACP, acyl carrier protein; *Cp*, *Cryptosporidium parvum*; *Et*, *Eimeria tenella*; NA, not applicable; *Pf*, *Plasmodium falciparum*; *Tg*, *Toxoplasma gondii*.
[b]D.S. Roos, unpublished.

gene from the apicoplast genome[1] suggests the *rpoD* gene has been relocated to the nucleus in apicomplexan parasites. The α-subunit gene, *rpoA*, is presumably also nuclear-encoded. The α_2, β, β′ polymerase of bacteria and plastids is highly sensitive to rifampicin and the antimalarial activity of rifampicin[22] suggests that this drug might block apicoplast transcription.

Little is known about RNA processing in the apicoplast and therefore its potential utility as a drug target is unclear. Many genes appear to be transcribed as operons but how (or if) these are processed is not known. RNA editing does not seem to occur, but the tRNA-Leu gene contains a group I intron[1]. Neomycin B and chlorotetracycline inhibit group I intron excision[23] and these classes of antibiotics might thus be worth testing against apicomplexan parasites.

Translation

Numerous antibacterial agents work by inhibiting protein translation. There is currently no direct proof of translation in the apicoplast (despite the efforts of many laboratories) but, based on indirect evidence, apicoplast genes are almost certainly translated. For example, the apicoplast genome contains the genes for ribosomal RNAs predicted to fold into proper ribosomal subunits, open reading frames predicted to encode many ribosomal proteins, translation components such as elongation factor-Tu, and a full set of tRNAs[1]. Persistence of these and other genes provides strong evidence for a translation system. Moreover, ribosome-like particles of bacterial size are visible in the apicoplast[2,12]. Finally, several drugs that block prokaryotic translation systems are parasiticidal[24]. Some of these drugs, including doxycycline, clindamycin and spiramycin, are used clinically for the treatment of malaria and toxoplasmosis, so it is vital that we understand how, or indeed whether, they affect the apicoplast.

Lincosamides (e.g. lincomycin and clindamycin) and macrolides (e.g. erythromycin and azithromycin) block protein synthesis by interacting with the peptidyl-transferase domain of bacterial 23S rRNA[25].

It has long been known that these antibiotics inhibit the growth of apicomplexan parasites such as *P. falciparum*, *T. gondii*, *C. parvum* and *Babesia microti* (reviewed in Refs 19,26). In *T. gondii*, there is strong circumstantial evidence of a blockage in apicoplast translation[24,27–29].

Two thiopeptide antibacterial agents, thiostrepton and micrococcin, are potent inhibitors of *P. falciparum* growth *in vitro*[30,31]. Indirect evidence strongly suggests this growth inhibition is a result of these antibiotics binding to the large subunit of rRNA and blocking apicoplast translation[32,33]. In *P. falciparum*, only the apicoplast rRNA is susceptible to thiostrepton and it is therefore unlikely that thiostrepton affects cytosolic or mitochondrial rRNAs. In *T. gondii*, the apicoplast rRNA is not susceptible to thiostrepton, and *T. gondii* growth is therefore not inhibited by the drug[33].

Several other inhibitors of bacterial translation, including tetracycline and doxycycline, are also parasiticidal and could inhibit plastid translation (Table 1), although the precise target of these drugs is uncertain[27,34].

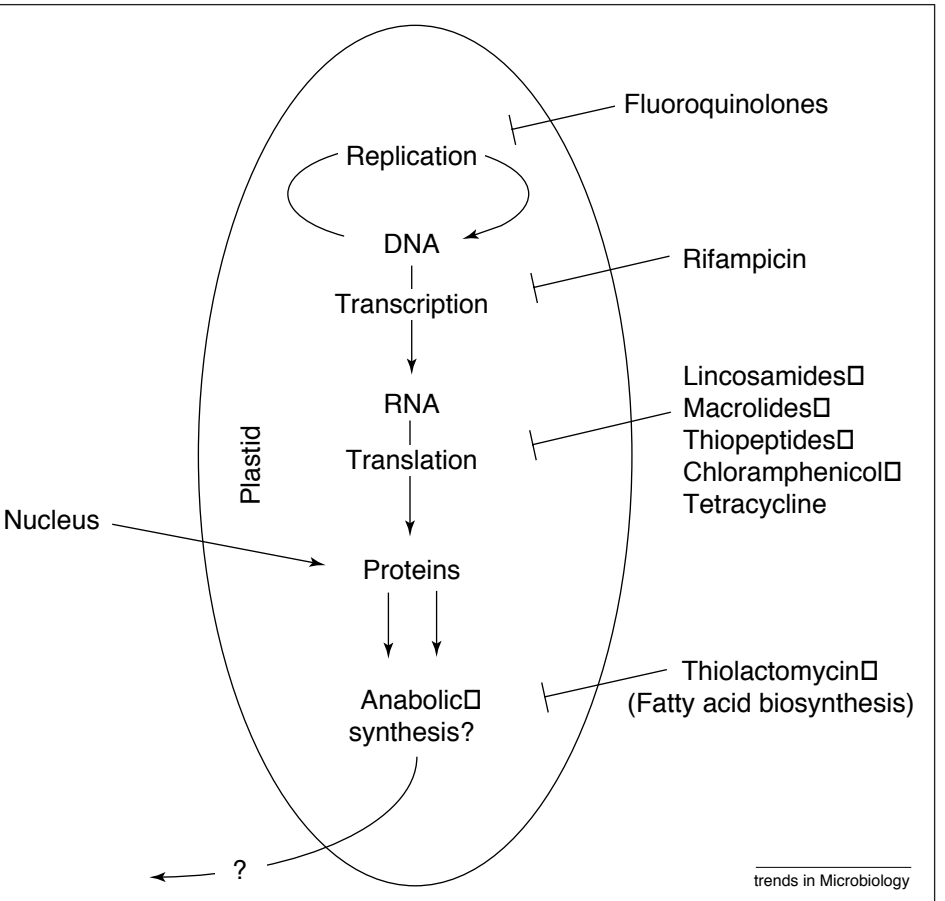

Fig. 2. Plastid metabolic pathways and the major classes of parasiticidal antibiotics believed to target these pathways. The plastid pathways are bacterial in nature and hence are sensitive to many antibacterial compounds. The ultimate function of the relict plastid is thought to be anabolic synthesis of some component(s), possibly a fatty acid or lipid[6], essential for cell function.

Targeting the ultimate function of the apicoplast

The apicoplast appears to be non-photosynthetic: it contains little or no pigment[9] and is completely devoid of the internal membranes (thylakoids) in which photosynthetic plastids perform light-energy-harvesting reactions[2,12] (Fig. 1). A lack of photosynthesis is consistent with the parasitic lifestyle of the Apicomplexa, much of which occurs in darkness. Why, then, has the plastid been retained? Our working hypothesis is that plastids supply some component or service to the parasite (Fig. 2). The plastids of plants and algae are the sites of several indispensable anabolic pathways. These plastid pathways, which include the biosynthesis of heme, aromatic amino acids and fatty acids, are homologous to cyanobacterial pathways, a fact that is consistent with their acquisition by endosymbiosis[35]. Plastid-containing cells have become dependent on these pathways for certain metabolic products that are exported from the organelle for use in the surrounding cytoplasm. This dependency makes plastids indispensable, even in non-photosynthetic plants and algae[5]. It is not clear whether the endosymbiont pathways replaced pre-existing cytoplasmic pathways or whether they were adopted as novel pathways not previously found in the hosts (which, as phagotrophs, might have been able to scavenge for their requirements)[35]. Many of the genes encoding proteins involved in these pathways have been relocated into the host nucleus but they retain clear traces of their plastid/cyanobacterial ancestry in gene phylogenies[36,37].

It is of paramount importance to determine which, if any, of these pathways are present in the apicoplast. The cyanobacterial nature of plastid pathways makes them very appealing as potential drug targets because they are fundamentally different from the equivalent pathways in animal hosts. For example, animals synthesize heme from glycine and succinyl CoA using the Shemin pathway, but plastids (and their cyanobacterial ancestors) use an unusual pathway[38] starting with glutamate ligated to tRNA-Glu. The Shemin pathway occurs in *P. falciparum*[39] (presumably in the mitochondrion and cytosol) but there is, at present, no evidence for a cyanobacterial heme pathway in the apicoplast[1].

Aromatic (essential) amino acids are synthesized by the shikimate pathway, which is located in the plastid in plants and algae. Animals lack a shikimate pathway, which is why aromatic amino acids are essential in their diet. *Plasmodium* has been presumed to be auxotrophic for aromatic amino acids, but this has recently been called into question with the

discovery of a shikimate pathway in *Plasmodium*, *Cryptosporidium* and *Toxoplasma*[40]. This could explain the parasiticidal activity of glyphosate, a common herbicide (RoundUp™, Zero™, Tumbleweed™), which blocks the activity of 5-enopyruvyl shikimate 3-phosphate synthase[40]. Although the shikimate pathway could be a useful target for parasiticidal drug design, these enzymes appear to be located in the parasite cytosol (as they are in fungi), rather than in the apicoplast[41].

Fatty acid biosynthesis is another essential function of plant and algal plastids. The plastid pathway is cyanobacterial in nature[42] and is very different from the eukaryotic, cytosolic pathway of animals and fungi[43]. Our laboratories have recently provided evidence for a plastid-associated fatty acid biosynthetic pathway in *Plasmodium* and *Toxoplasma*[6]. Again, it had been thought that *Plasmodium* was auxotrophic for fatty acids, but our data suggest the presence of a previously overlooked pathway. A selective inhibitor of plastid-type fatty acid biosynthesis shows activity against both *Plasmodium* and *Toxoplasma in vitro*, suggesting that this pathway could, indeed, be a useful drug target[6].

Conclusions

A wide range of well-characterized drugs and herbicides are suspected to block activity of the apicomplexan plastid (Table 1). All aspects of apicoplast function, including DNA replication, transcription, translation and anabolic synthesis, can potentially be blocked by one or more known compounds from the existing pharmacopeia of drugs and herbicides (Fig. 2). Interestingly, blocking these plastid activities does not usually result in immediate parasite death. In *Toxoplasma*, for example, the parasiticidal activity of plastid inhibitors is only seen when tachyzoites attempt to establish infection inside a new host cell[17,24,28]. A similar 'delayed-death' phenotype is observed in *Plasmodium*[14]. These observations might indicate that plastids assemble a pool of some component(s) essential for the formation of the intrahost compartments known as parasitophorous vacuoles. At present, our favored hypothesis is that the apicoplast synthesizes specific fatty acids or other lipids required for successful establishment of the parasitophorous vacuole during invasion of a new host cell[6]. More work is needed to substantiate the existence and role of plastid anabolic pathways in apicomplexan parasites, and to validate these enzymes as targets for parasiticidal drugs – but it is already clear that this area holds considerable promise for rational drug design.

Questions for future research

- Do all 5000 species of apicomplexan parasites contain a plastid?
- How did apicomplexan species acquire this organelle?
- What is the function of the unidentified open reading frames in the apicoplast genome?
- Which nuclear-encoded proteins are targeted into the plastid? How many of these are there, and do they include any metabolic pathways not normally associated with plastids?
- Were any non-plastid genes transferred from the algal nucleus engulfed during secondary endosymbiosis to the parasite nucleus?
- Does the apicoplast synthesize fatty acids and/or other lipids?
- Is the shikimate pathway in the Apicomplexa located in the plastid or the cytosol?
- Why does interference with plastid function cause delayed death rather than an immediate effect?

Note added in proof

The origins, targeting and possible functions of the apicoplast are discussed in Roos, D.S. *et al.* (1999) *Curr. Opin. Microbiol.* 2, 426–432.

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Regulatory networks controlling *Candida albicans* morphogenesis

Alistair J.P. Brown and Neil A.R. Gow

Candida albicans **undergoes reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms that promote the virulence of this pathogenic fungus. The regulatory networks that control morphogenesis are being elucidated; however, the primary signals that trigger morphogenesis remain obscure, and the physiological outputs of these networks are complex.**

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Candida albicans is the major fungal pathogen in humans, particularly in immunocompromised patients[1]. This fungus can colonize and infect a wide range of microenvironments in the body including the bloodstream, superficial sites in the mucosa and all of the major internal organs during systemic disease. *C. albicans* is pleiomorphic and undergoes reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms. Pseudohyphae range from relatively short to extended cells and, unlike hyphae, they have constrictions at their septa[2]. The developmental relationship between pseudohyphae and hyphae has not yet been established and there are clear differences in the cell cycle between these forms; however, many view pseudohyphal cells as intermediates between the morphological extremes represented by yeast and hyphal cells.

Morphogenesis is triggered by various signals *in vitro*. Many of the responses to these signals probably reflect normal interactions between the fungus and its host *in vivo*. The yeast-to-hypha transition has now been shown to be one of several virulence attributes that enable *C. albicans* to invade human tissues (Fig. 1). Hyphal growth is blocked by inactivation of the transcription factors Cph1p and Efg1p (Ref. 3), which belong to the mitogen-activated protein kinase (MAPK) and Ras–cAMP pathways described below. Significantly, a *cph1 efg1* mutant also displays reduced virulence in animal models of systemic candidosis[3]. It is not clear what other virulence attributes might be regulated by these transcription factors.

Systemic models of *Candida* disease have been used extensively in recent years as a convenient way of evaluating the roles of individual genes in pathogenesis[3–5]. Immunocompromised or immunocompetent animals are normally injected in the tail vein and death curves are plotted after inoculation with similar numbers of congenic parental and mutant *Candida* strains. This simple laboratory test is an appropriate model of infection via central venous catheters. However, it rarely reflects the epidemiology of natural systemic *Candida* infections, which progress from a colonized mucosal surface in the gut, alimentary canal or vaginal tract to the 'deep' organs through a number of epithelial and endothelial barriers. Hyphae may be suited to breaching such barriers but yeast cells might be disseminated more effectively. In general, yeast cells predominate during mucosal colonization in the normal host but more hyphae emerge as the host defences decline. Hence, both growth forms could play important roles in pathogenesis and they both encounter many different microenvironments in the host. Similarly, evidence is emerging that the pathway of disease progression involves a transcriptional programme linking morphogenesis with other virulence traits such as adherence and aspartyl-proteinase production[6]. Here, we attempt to extend the current perspectives of morphogenetic signalling pathways, which do not fully account for the temporal and spatial complexity of their inputs (signals) and outputs (responses).

Inputs: signals triggering morphogenesis

A wide range of *in vitro* culture conditions and an even greater range of specific biochemicals have been shown to influence the yeast-to-hypha transition in *C. albicans*[1]. Because hypha formation is eliminated in *cph1 efg1* mutants[3], it appears that a multiplicity of

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