Part Three
Role of Host Cell Kinomes and Phosphatomes in Parasitic Infections

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9
Hijacking of Host Cell Signaling by *Theileria*

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Abstract
The apicomplexan parasites *Theileria annulata* and *T. parva* possess the ability to transform the infected host cell and induce uncontrolled proliferation. Residing free in the cytosol of its host leukocyte, the schizont is in a perfect position to manipulate host cell signaling pathways involved in regulating apoptosis, proliferation, and cell motility. While extensive *Theileria*-induced changes in host cell protein phosphorylation patterns have been reported, no *Theileria*-encoded kinases or phosphatases have been demonstrated - or are even predicted - to be associated with the schizont surface or secreted into the host cell. Instead, it seems that *Theileria* has evolved the capacity to modulate kinases of the host cell. In certain cases this involves "hijacking" pivotal kinases, such as the IκB kinase complex or the mitotic kinase polo-like kinase 1, recruiting them to the schizont surface. In this chapter the current understanding of *Theileria*-induced changes in host cell kinase activation is reviewed, and an attempt is made to link these events to phenotypic changes that occur in the cell in response to *Theileria* infection.

Introduction

Transforming species of the *Theileria* genus cause severe lymphoproliferative disease of cattle. Transmitted by ticks, *T. annulata* causes tropical theileriosis in Northern Africa, the Middle East and large areas of Asia, whereas *T. parva* infection results in East Coast Fever, which is prevalent in sub-Saharan East, Central, and Southern Africa.

Theileria is unique among the Apicomplexa in that it possesses the ability to "transform" its host cell, inducing uncontrolled proliferation, resistance to apoptosis and increased cellular migration and metastasis, conferring a malignant phenotype to the parasitized cell (for reviews, see Refs [1–3]). *Theileria parva* targets T and B lymphocytes, while *T. annulata* infects monocytes/macrophages and B cells [3]. *Theileria*-transformed cells can migrate into different tissues to establish new

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foci of infection and it is this metastatic potential, combined with the unchecked proliferation of parasitized leukocytes and the massive inflammatory responses that they induce, which leads to severe pathology.

Entry and development of *Theileria* in the host cell differs from that of other Apicomplexa. Instead of modifying the endocytic membrane that surrounds the parasite to form a parasitophorous vacuole, immediately after entry the invading sporozoite escapes lysosomal destruction by eliminating the surrounding plasma membrane and establishes its niche within the host cell cytoplasm. There, the liberated sporozoite rapidly associates with host cell microtubules, an interaction that is maintained throughout the intracellular stage. The sporozoite subsequently undergoes multiple rounds of genomic replication and karyokinesis, resulting in a large, multinucleated syncytium called a schizont [4].

While it is well established that many viruses and some bacteria can transform their host (or bystander) cells [5], *Theileria* is the only eukaryote known to do so. Importantly, host cell transformation strictly depends on the presence of the schizont and is reversible upon parasite removal, indicating that parasite-induced changes to the host are of an epigenetic nature. Drug-induced elimination of the parasite halts host cell proliferation, and macrophages cured of *T. annulata* infection recover at least some of the macrophage-specific features and functions that were lost upon infection.

A recent transcriptome analysis indicates that the parasite establishes tight control over host cell pathways associated with cellular activation, proliferation and survival, resulting in significantly altered patterns of gene expression likely to be beneficial to the establishment and persistence of the transformed cell [6]. Kinases are key regulators of signaling pathways that control gene expression. The activation of several transcription factors including nuclear factor kappaB (NF-κB), AP-1, ATF-2, c-Myc and STAT3 has been attributed to altered kinase signaling activity in *Theileria*-transformed leukocytes. In this chapter, an attempt is made to link these events to the phenotypic changes that result from this fascinating host–parasite interaction (as summarized in Figure 9.1).

Early studies on *T. annulata* revealed extensive changes in host cell protein phosphorylation patterns [7] and a four- to 12-fold increase in total protein phosphorylation could be observed in *T. parva*-infected T cells compared to uninfected T cells [8]. In this context, it was perhaps counterintuitive to find that genes encoding kinases or phosphatases predicted to be either secreted or surface-expressed by the schizont are lacking in the *Theileria* genomes [1,9,10]. This is in contrast to other apicomplexan parasites such as *Toxoplasma gondii*, which has been shown to secrete ROP kinases into the host cells to directly modulate nuclear processes. An example is ROP38, which downregulates host genes associated with MAPK signaling and the control of apoptosis and proliferation [11]. *Theileria* parasites have clearly evolved other means of manipulating host cell signaling pathways and modulating the activity of regulatory host-cell kinases.

Four important characteristics of *Theileria*-induced transformation rely on the modulation of host cell kinases, and these will form the subject of this chapter: (i) resistance to apoptosis; (ii) uncontrolled proliferation; (iii) increased motility and metastatic potential; and (iv) persistence in a continuously dividing cell.

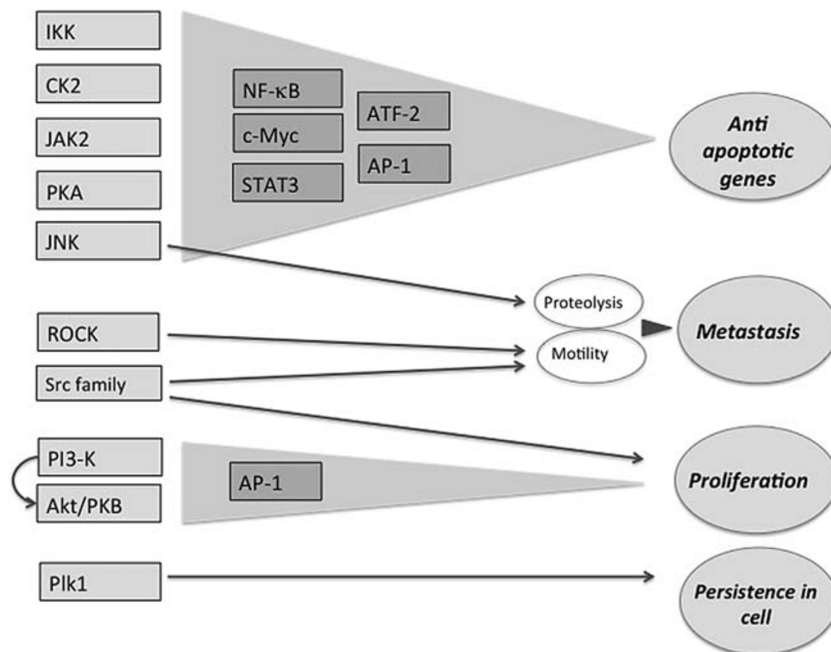

Figure 9.1 Parasite interference with host cell signaling pathways in *Theileria*-infected cells. The constitutive activation of IKK (in the form of "signalosomes" at the parasite surface), CK2, JAK2, JNK and PKA have all been implicated in conferring protection against apoptosis to *Theileria*-transformed cells, via the activation of various important transcription factors (including NF-κB, c-Myc, STAT3, ATF-2, and AP1). Rho family kinase (ROCK) and Src family kinases are involved in promoting polarization and motility of *Theileria*-infected cells, while JNK activity contributes towards the proteolytic potential of infected cells. Constitutive PI3-K, Akt/PKB and Src family kinase activity is dispensable for protection against apoptosis, but is required to maintain a continually proliferative state. Plk1 is recruited to the parasite surface, and its activity is required for successful parasite segregation during host cell cytokinesis.

Theileria-Induced Resistance to Apoptosis

Uncontrolled or unscheduled cellular proliferation often elicits apoptotic triggers aimed at eliminating the "unruly" cell. To protect against this, a range of antiapoptotic proteins including c-FLIP, c-IAP1, c-IAP2, X chromosome-linked IAP, and the Bcl-2 family member Mcl-1, are upregulated in *Theileria*-transformed lymphocytes, and cells show increased resistance against signaling via the death receptors TNFα or Fas [12–15]. Several host cell kinases have been implicated in the defense against apoptosis.

Hijacking of IKK Signalosomes by *Theileria*

The transcription factor NF-κB controls the expression of a panel of genes involved in immune response, inflammation, proliferation, and survival (for a review, see

Ref. [16]). In *Theileria*-transformed cells the transcription factor NF-κB is constitutively activated, protecting the cell against apoptosis [17]. NF-κB activation is normally controlled by the IKK complex, a multisubunit kinase complex consisting of two catalytic subunits (IKK1 and IKK2), and a modulating unit NEMO (NF-κB essential modulator, also called IKKγ). *Theileria* employs a novel mechanism to ensure NF-κB activation, whereby IKK is aggregated in large, activated "signalosomes" at the parasite surface [18]. Here, IKK phosphorylates IκB-α and IκB-β, two cytoplasmic inhibitors of NF-κB, tagging them for ubiquitination and proteasomal degradation. In the absence of these inhibitors, NF-κB translocates into the nucleus of the parasitized cell and activates NF-κB-dependent transcription. In contrast to many other IKK activation pathways, parasite-induced IKK activity does not require functional Hsp90 [19]. By bypassing upstream activation steps, parasite-induced IKK activation not only gains independence from surface receptor-mediated signaling but, in all likelihood, also escapes the downregulatory mechanisms known to act upstream of IKK [18]. Signaling via surface receptors appears not to be blocked in general, however, as *Theileria*-transformed cells can still respond to autocrine signals with increased NF-κB activity [14].

The identity of the parasite protein binding IKK is still unknown. The formation of parasite-associated IKK signalosomes was shown to be influenced by the architecture of the actin cytoskeleton [20]. Disruption of the latter using cytochalasin resulted in increased IKK signalosome and NF-κB activity. A *T. parva*-specific schizont surface protein, TpSCOP, which is capable of binding host cell F-actin, was proposed to play a role in NF-κB activation [21]. While no direct interaction between TpSCOP and components of the IKK signalosome could be demonstrated, it is possible that TpSCOP contributes to NF-κB activation via interaction with the host cell cytoskeleton.

Functional CK2 is Required for Maintaining *Theileria*-Induced Transformation

The serine/threonine-specific casein kinase 2 (CK2), a pleiotropic enzyme known to phosphorylate several hundred cellular substrates, is upregulated and activated in *Theileria*-transformed cells [8] and is largely responsible for the dramatic increase in phosphorylation in response to *Theileria* infection. Inhibition experiments – perhaps not surprisingly – confirmed the importance of CK2 in the survival and proliferation of *Theileria*-transformed cells [22–24].

c-Myc is a transcription factor that, typically, is constitutively expressed in many cancer cells and can act as both a transcriptional activator and repressor [25]. The constitutive activation of c-Myc is a key feature of *Theileria*-induced transformation, and inhibition of c-Myc in *Theileria*-infected lymphocytes leads to a loss of expression of the antiapoptotic protein Mcl-1, and the onset of apoptosis within 24 h [12,26]. One of the important functions of CK2 is to prolong the half-life of the transcription factor c-Myc by reducing its proteasomal degradation [12,27]; this is achieved by phosphorylation of the c-Myc C-terminal PEST domain. CK2 also contributes to NF-κB activation in *T. parva*-transformed B cells, in this case by promoting proteasomal degradation of the inhibitor IκB [22]. As NF-κB contributes

to c-Myc expression, CK2 thus functions at both the transcriptional and post-translational level to promote the continuous activation of c-Myc in parasitized cells. CK2 is also involved in the suppression of caspase 3 activity, adding yet another level of protection against apoptosis [22].

Mitogen-Activated Protein Kinases (MAPKs): The Activation of JNK

MAPKs encompass the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 kinases. JNK is a stress-activated protein kinase that is constitutively activated in a parasite-dependent manner [28], where it contributes to the activation of transcription factors ATF-2 and AP-1 [29,30]. Parasite interference with MAPK pathways appears to be restricted to the constitutive activation of JNK, as ERK1/2 and p38 were found to be inactive in *Theileria*-transformed cell lines [28,30]. However, a later study reported that while ERK1 was inactive in all clonal cell lines, varying levels of ERK1 activity could be observed in bulk cultures [31]. The exact reason for this discrepancy is not known, but one possibility might be the presence in bulk cultures of uninfected cells in which ERK was activated.

Although IKK and JNK activation is often coregulated, there is no evidence for the direct activation of JNK at the schizont surface, and the way(s) in which JNK is (are) activated in *Theileria*-transformed cells is still not known. Signals emanating from surface receptors on the *Theileria*-transformed cell probably contribute to constitutive JNK activation. In this context, it has been proposed that autocrine stimulation of the receptors for tumor necrosis factor alpha (TNFα) [14] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [32] contribute to AP-1 stimulation. AP-1 activity is also dependent on Src-family kinase activation [33], but whether this pathway also feeds into the JNK activation pathway has not been demonstrated.

Depending on the stimulus and cellular context, JNK can have either proapoptotic or antiapoptotic effects (for a review, see Ref. [34]). In *T. parva*-transformed B cells, the overexpression of dominant negative mutant forms of JNK, or treatment with a JNK inhibitor, results in apoptosis [35]. The inhibition of c-Jun (a subunit of AP-1 and a target of JNK) *per se* did not induce apoptosis, but was nevertheless found to result in a downregulation of antiapoptotic Mcl-1 and c-IAP and a sensitization of the infected cells to the effects of BW-270c killing of the parasite [35]. Thus, JNK activation and c-Jun induction have overlapping – but not necessarily identical – antiapoptotic roles in *Theileria*-transformed B-cells.

JAK2 Kinase

STATs (signal transducers and activators of transcription) are transcription factors that are phosphorylated and activated in response to cytokine- and growth factor-mediated signaling, with STAT3 and STAT5 often being permanently activated in malignant tumors [36]. In *T. parva*-infected B cells, STAT3 is constitutively phosphorylated in a parasite-dependent manner by the

Janus activated kinase 2 (JAK2), and killing of the parasite results in a gradual reduction of STAT3 phosphorylation [26]. STAT3 regulates the expression of many genes important for proliferation and survival, including cyclin D1, c-Myc, Bcl-xL, and Mcl-1. The inhibition of JAK2 in infected B cells results in a rapid dephosphorylation of STAT3, and causes a significant drop in c-Myc levels. Cotransfection reporter assays have shown clearly that JAK2-STAT3 signaling is involved in c-Myc activation which, in turn, is required for expression of the antiapoptotic protein, Mcl-1.

The difference in kinetics of STAT3 dephosphorylation caused by parasite death (48–72 h) and that caused by JAK-2 inhibition (6 h) suggests that parasite-dependent JAK-2 activation is indirect, and potentially involves JAK-2 linked cytokine receptors. The latter are triggered by cytokines, which are released by the transformed cells and remain in the supernatant for some time after parasite elimination. In this context, it has been proposed that a *Theileria*-dependent GM-CSF autocrine loop is responsible for activating JAK2-STAT3 which, in turn, plays a role in c-Myc transactivation [12]. However, the finding that parasite elimination provokes a rapid drop in c-Myc levels which occurs before any detectable loss in STAT3 phosphorylation [12] indicates that the transcription of c-Myc does not depend solely on the JAK2–STAT3 pathway.

A PKA-Dependent Survival Pathway

The infection of a transformed B-cell line (BL3) with *T. annulata* is accompanied by increased levels of PKA activity involving the upregulation of its β catalytic subunit [37]. Inhibition studies point towards the existence of a PKA-dependent antiapoptotic survival pathway. This appears to linked to phosphatidylinositol-3-kinase (PI3-K), but the exact organization of the pathway has not yet been elucidated. Chemical PKA inhibition also induces apoptosis in *T. parva*-transformed T- and B-cell lines, suggesting that a PKA survival pathway could be a general feature of *Theileria*-infected lymphocytes.

Increased Proliferation

Phosphatidylinositol-3-Kinase (PI3-K)

Phosphoinositide kinases play important roles in many cellular processes, including proliferation and survival [38]. PI3-K is constitutively upregulated in a parasite-dependent manner in all *Theileria*-transformed cell lines studied [32,39,40]. PI3-K activity was found to be crucial for proliferation, as an inhibitory treatment of cells with LY294002 or wortmannin results in an impaired S-phase entry, most likely caused by a loss of activation of the downstream p70/S6 kinase. Perhaps surprisingly, PI3-K inhibition was not accompanied by a pronounced increase in apoptosis, which suggests that these processes are uncoupled in *Theileria*-transformed cells. In *T. parva*-transformed B cells, proliferation is

augmented through a GM-CSF autocrine loop that involves sustained activation of PI3-K and induction of AP-1 [32].

PI3-K was also proposed to contribute to enhanced transferrin-receptor (TfR) expression observed in *T. parva*-transformed B cells [41], as both surface expression and protein levels of TfR are downregulated after the inhibition of PI3-K. As TfR expression is known to be regulated by AP-1 [42], and PI3-K was found to contribute to constitutive AP-1 induction in infected cells, it was suggested that the regulation of TfR levels by PI3-K in *Theileria*-infected cells is mediated via the constitutive induction of AP-1 [41]. In a further study, a potential AP1 binding site was identified in the Rab11a promoter (REF 43). JNK also regulates AP1 activity [28,30], and pharmacological inhibition of the JNK pathway reduced Rab11 protein levels and endosome recycling of the TfR; furthermore, siRNA knockdown of JNK1 levels reduced TfR surface expression [43]. Whether PI3K and JNK function in the same signaling pathway was not directly determined, however.

Akt/PKB

The serine/threonine kinase PKB (Akt) is a central downstream effector of PI3-K. Akt/PKB is constitutively activated in a PI3-K-dependent manner in *T. parva*-transformed T and B cells, and in *T. annulata*-transformed TaC12 cells [40]. In a separate study, Akt/PKB activation (as measured by Ser473 phosphorylation) was not demonstrated in *T. parva*-infected B cells [32], though this was most likely due to differences in experimental procedure. The expression of an inactive form of Akt/PKB in *T. parva*-transformed T cells resulted – presumably through a dominant-negative mechanism – in a marked decrease in DNA synthesis [40], confirming that Akt/PKB contributes to proliferation. Akt/PKB activity is downregulated with slow kinetics upon parasite killing, suggesting that parasite-mediated Akt/PKB activation is indirect and is more likely mediated via surface receptor activation. A link between Akt/PKB and activation of the IKK complex, as reported by others [44], could not be confirmed for *Theileria*-transformed cells, and Akt/PKB activity is not required for NF-κB-mediated protection against apoptosis. Akt/PKB activity could be further induced by treatment with phosphatase inhibitors [40].

Interestingly, in a spontaneously transformed B-cell line (BL3) subsequently infected with *T. annulata* (TBL3), levels of Akt/PKB activation (as measured by Akt/PBB Ser473 phosphorylation) were undetectable [37]. How, in this case, the transforming events that had occurred prior to infection would interdigitate with a *Theileria*-induced reprogramming of the cell was not clear, but these observations underpinned the notion that high levels of Akt/PKB activity are not required to maintain the transformation and survival of these cells.

Src Family Kinase, Hck

Src family kinases are involved in transducing signals that emanate from a wide range of different surface receptors, including growth factor receptors. They are widely expressed, but differ in their expression patterns depending on the cell type.

Activated Src-related kinases such as Fyn, Lck and Hck can be detected in different *Theileria*-transformed cell lines [33,45,46], and inhibitors of these enzymes block proliferation.

Studies in *T. parva*-transformed B cells have shown that, similar to PI3-K, Hck activity is not needed for protection against apoptosis [33]. Instead, Hck – most likely in conjunction with PI3-K – was found to contribute towards complete AP-1 activation. Src family kinases are found in glycolipid-enriched microdomains (GEMSs; also known as "lipid rafts"), where they are negatively regulated by the tyrosine kinase Csk that forms a complex with the transmembrane adapter protein PAG (phosphoprotein associated with GEM). In *T. parva*-transformed B cells, constitutive Hck activity is mediated by the exclusion of Csk from lipid rafts [33].

Increased Metastatic Potential

Theileria-transformed cells possess the ability to migrate across tissue barriers and to disseminate throughout the host animal, establishing multiple foci of proliferation. This metastatic behavior contributes to the pronounced pathology observed in susceptible animals. Cell motility is intrinsically linked to their polarization, and the mechanisms by which *Theileria* manipulates and polarizes its host cells have recently been reviewed [2]. As would be expected, the activity of several host cell kinases are intricately involved in this process.

Src Family Kinases

In addition to promoting proliferation, the Src family kinase Hck also contributes to polarization of the parasitized host cell by manipulating host cell actin dynamics. This is particularly evident in adherent cell lines [47]. *T. annulata*-transformed macrophages exhibit a reduced "random motility" and possess a very different morphology compared to their cured counterparts. Infected cells possess a single, actin-rich lamellipodium at their leading edge as well as multiple "podosome-type adhesions" (PTAs), whereas uninfected cells possess no polarized lamellipodia and have significantly fewer PTAs REF 47. Lamellipodia and podosomes are actin-based cellular structures that are intricately involved in regulating macrophage motility. Lamellipodia are sheet-like protrusions which are found at the leading edge of migrating cells and provide the driving force required for motility. Podosomes are short-lived column-like adhesion structures that are structurally similar to "invadopodia," and are associated with high levels of proteolysis and the invasive behavior of tumor cells. The presence of lamellipodia and PTAs correlates with a matrix-degrading ability, suggesting a possible role in proteolysis and invasiveness [48]. Activated Src kinases (mainly Hck and c-Src) accumulate in the lamellipodia of parasitized cells, and the extension of persistent lamellipodia was found to depend on Src-kinase activity. In parasitized cells, Hck activity was required for the accumulation of ERM (ezrin, radixin, moesin) family proteins, which are known to regulate actin dynamics and stability by linking transmembrane proteins to the

actin cytoskeleton in the cortex, at cortactin-rich lamellipodia. Thus, in *Theileria*-transformed cells, host cell Src family kinases participate in altering adherence and invasion properties, and potentially contribute to the dissemination and virulence of this parasite [47].

In the same context, the marked upregulation of *PAK1* gene expression in *Theileria*-infected BL20 cells [6] observed by microarray analysis could be of interest. *PAK1* encodes a serine/threonine kinase that has been implicated in tumor progression. It is a key regulator of the actin and microtubule cytoskeleton and, by phosphorylating cortactin, it plays a crucial role in podosome formation and membrane ruffling.

Rho Kinase (ROCK)

Rho kinase (ROCK) is involved in cytoskeleton remodeling, and was recently implicated in conferring a metastatic potential to *Theileria*-transformed cells [49]. In live vaccines, attenuation is associated with a reduced invasiveness of infected leukocytes *in vitro* which, in turn, appears to be linked to reduced levels of TGF-β2. Interestingly, infected macrophages from disease-susceptible cattle (*Bos taurus*, Holstein-Friesian) produce more TGF-β2 and traverse Matrigel with a greater efficiency compared to those from disease-resistant cattle (*Bos indicus*, Sahiwal). TGF-β2-associated invasiveness requires ROCK activation, and pharmacological inhibition of the kinase results in an impaired cell motility in three-dimensional matrices.

JNK Activation Contributes to Metastatic Potential

JNK kinase is activated in *Theileria*-transformed cells, and contributes not only to protection against apoptosis (as previously discussed) but also to promoting the metastasis of infected lymphocytes [35]. In addition to movement and migration, the process of metastasis requires the degradation of extracellular matrix components. The metastatic potential of *T. annulata*-infected macrophages correlates with AP-1-dependent induction of metalloprotease-9 [50]. JNK can participate in this process by phosphorylating and activating c-Jun which, together with c-Fos, forms AP-1. Furthermore, it could be shown in *T. parva*-transformed B cells that a reduction in c-Jun expression would result in reduced synthetic matrix degradation *in vitro*, and also would impair tumor formation in mice [35]. Taken together, the results of these studies point to an important role for JNK signaling in mediating the metastatic potential of *Theileria*-transformed cells.

Persisting Within a Dividing Cell

Theileria schizonts are strictly intracellular, and their presence is absolutely required to maintain the transformed state of the infected host cell. Mammalian cell mitosis is a highly regulated process involving the precise spatiotemporal contribution of a broad set of proteins, including several kinases such as Cdk1,

Plk1, and Aurora kinases. These coordinate the correct timing and fidelity of processes such as centrosomal functions, spindle assembly and microtubule-kinetochore attachment, as well as sister chromatid separation and cytokinesis (for a review, see Ref. [51]). Importantly, as mitotic failures often result in apoptosis, it is essential that the parasite avoids interference with this finely tuned process.

Schizont DNA synthesis occurs predominantly during late G_2 and early M phase of the host cell [52]. Schizont segregation is a passive process that relies on host cell cytokinesis. Recently, a detailed investigation showed that an interaction with both central spindle and astral microtubules is required for successful parasite segregation [53]. How the parasite, once again, usurps a host cell kinase is outlined in the following subsection.

Hijacking of Host Cell Plk1

Polo-like kinase 1 (Plk1) is one of the key regulators of mammalian mitosis, playing important roles during mitotic entry, centrosome maturation, bipolar spindle formation, chromosome segregation, and finally in regulating cytokinesis [54]. Plk1 activity is carefully regulated in a spatial and temporal manner, and in keeping with its divergent roles, shows a dramatic cell cycle-dependent localization. The kinase localizes to centrosomes during G_2 phase, to kinetochores and spindle poles during prometaphase and metaphase, and finally to the central spindle and mid-body during anaphase and telophase. Plk1 localization and activity is regulated by the phosphorylation status of its docking partners. Plk1 docking normally relies on the binding of its "polo-box domain" (PBD) to the phosphorylated motif S-(pS/pT)-(P/X) [55]. In many cases (e.g., Nedd1, BubR1, PICH, Cep55, INCENP, all of which are Plk interactors) binding is regulated by Cdk1, which acts as the "priming kinase" [56], but in other cases Cdk1-mediated phosphorylation prevents Plk1 docking. For example, the phosphorylation of PRC1 by Cdk1 during metaphase prevents Plk1 binding, and only when Cdk1 activity decreases at the onset of anaphase can Plk1 bind to this protein, in this case by creating its own docking site through "self-priming" [57]. Thus, while Cdk1 usually generates docking sites for Plk1 during prometaphase and metaphase, Plk1 itself can take over with the onset of anaphase and create its own docking sites.

It was reported recently that host cell Plk1 binds to the schizont surface in a cell cycle-dependent manner [53]. Plk1 can be detected at the parasite surface during G_2 phase, but is notably absent during prometaphase and metaphase – when Cdk1 activity is high – and reassociates with the schizont as soon as cells enter anaphase (see Figure 9.2). The negative correlation between Cdk1 activity and parasite/Plk1 interaction was unexpected, but could be confirmed using the Cdk1 inhibitor RO-3306. Cdk1 inhibition resulted in a dramatic and immediate recruitment of Plk1 to the parasite surface which ruled out Cdk1 as the priming kinase. Plk1 binding does not depend on "self-priming" either, however, as a kinase-dead Plk1 mutant can still bind to the parasite surface, and chemical inhibition of Plk1 kinase activity actually enhances – rather than inhibits – Plk1-parasite binding. The priming kinase in this instance remains to be elucidated.

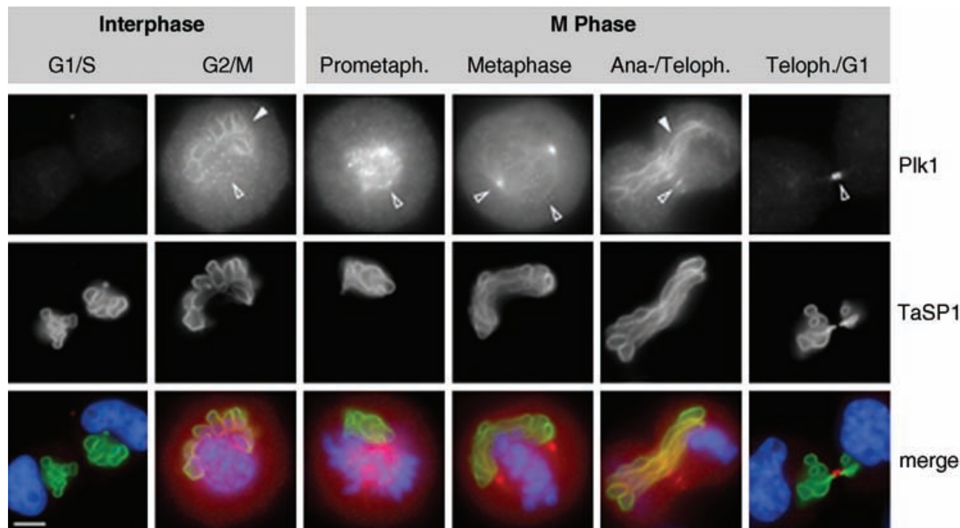

Figure 9.2 Biphasic recruitment of host Plk1 to the schizont surface. During G_2, when Plk1 is abundantly expressed, prominent labeling of the schizont surface with an anti-Plk antibody can be observed (closed arrows). Binding to the schizont was maintained until nuclear accumulation of cyclin B1 and nuclear envelope breakdown becomes apparent during prophase. Once cells reach prometaphase and metaphase, Plk1 localizes to spindle poles and kinetochores (open arrows), but is not associated with the schizont. With the onset of anaphase, Plk1 reaccumulates on the parasite surface. In cells progressing to telophase, Plk1 association with the parasite is largely restricted to the section of the schizont that is incorporated into the central spindle. The parasite surface is labeled with anti-TaSP1 (green), and host cell Plk1 using anti-Plk1 (red). DNA was stained with DAPI. Scale bar = 5 μm. Reproduced with permission from Ref. [53]; © 2010 C. von Schubert *et al*.

Plk1 binds to the schizont via its C-terminal PBD, and binding depends strictly on two critical PBD residues that mediate phospholigand binding; this implies the involvement of a serine/threonine kinase. However, the binding of Plk1 to the parasite is clearly "atypical" as neither Cdk1 nor Plk1 acts as the priming kinase. This conundrum is presently being investigated.

Initial findings have shown that the parasite surface is extensively phosphorylated in a cell cycle-dependent manner. While anti-pThr-Pro antibodies strongly stain the surface of the parasite during interphase and late telophase, no pThr-Pro or pSer-Pro staining can be observed throughout mitosis until late telophase. This suggests that a proline-directed Ser/Thr kinase is not directly involved in Plk1 recruitment to the parasite during anaphase and early telophase, and argues against Cdk1 phosphorylation preventing Plk1 binding in a mechanism similar to PRC-1 [57]. Interestingly, antibodies that recognize p-Thr epitopes weakly labeled the parasite following a temporal pattern that resembles that for Plk1 recruitment to the parasite (Figure 9.3), and suggesting that Plk1 recruitment might be regulated by timely Thr phosphorylation of the parasite surface. To date, Plk1 and

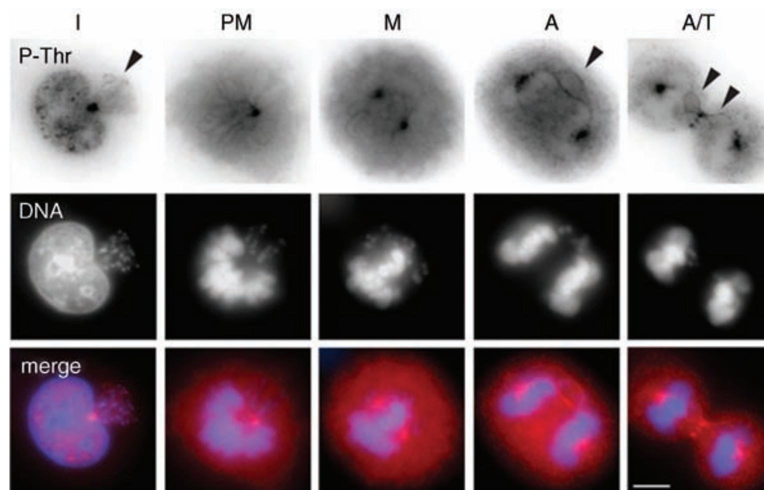

Figure 9.3 Threonine phosphorylation of the parasite surface during interphase and anaphase. During interphase (likely G_2 phase) the parasite surface is weakly labeled with p-Thr epitopes (indicated with arrow heads). Phosphorylation of the parasite surface was undetected during prometaphase and metaphase, returning with the onset of anaphase. Thus, p-Thr labeling of the parasite surface correlates with that of Plk1 recruitment (compare to Figure 9.2).

Cdk1 are the only kinases reported to "prime" Plk1 binding partners. The discovery of an "unusual" *Theileria* binding partner and/or substrates of Plk1 could provide important new insights in the regulation of this kinase.

The question of the significance of Plk1 recruitment to the parasite surface remains. It could be shown that Plk1 is required for the recruitment of the host cell central spindle to the parasite surface, but not for its interaction with astral MTs. Central spindle function and the successful completion of cytokinesis (including furrow ingression and abscission) are regulated by Plk1. A central spindle-localized pool of the RhoGEF Ect2 stimulates activation of the small GTPase RhoA, which drives contractile ring assembly at the equatorial cortex [58]. By assembling the central spindle at its surface, the schizont – true to its opportunistic-but-pragmatic nature – not only determines where furrow ingression takes place but also becomes "incorporated" in the process without perturbing furrow ingression and cytokinetic abscission.

When the interaction of the parasite with both central spindles (Plk1-dependent) and astral MTs (non-Plk1-dependent) is disrupted, parasite segregation no longer occurs in an equal manner, and the forming daughter cells often completely lack a parasite.

Thus, in order to ensure its persistence in a continuously dividing cell, *Theileria* usurps a key mitotic kinase. Considering the prominent role of Plk1 at different stages of mitosis, it is surprising that correct mitosis and cytokinesis are not

impaired. Perhaps the temporary Cdk1-dependent dislocation of Plk1 from the parasite surface during mitosis serves to guarantee a sufficiently large pool of Plk1 to fulfill important cellular tasks during prometaphase and metaphase, required for satisfying the spindle checkpoint.

Conclusions

Most other intracellular parasites have evolved elegant and efficient ways in which to outsmart the host's immune system and manipulate the infected host cell to their own advantage, securing survival and persistence. *Theileria* goes one important step further, and also induces an uncontrolled proliferation of its host cell. Pathogens tend to target host cell proteins that are hubs (those involved in many interactions and which allow crosstalk between several distinct pathways) or bottlenecks (those central to many paths in a network) [59]. By hijacking PLK1 [53] and IKK [18], two kinases with central and distinct roles in vital cellular processes, *Theileria* provides two striking examples of this strategy. *Toxoplasma gondii* infection also leads to the activation of NF-κB and upregulation of host cell antiapoptotic genes, including the Bcl-2 and IAP families, [60,61]. In this case, a kinase of parasitic origin appears to phosphorylate IκB at the parasitophorous vacuole membrane, leading to constitutive NF-κB activation [62]. This strategy has been selected by a large number of very diverse intracellular pathogens, including viruses: in many cases of viral infection, NF-κB is activated by viral oncoproteins. For example, both the ks-vFLIP protein of Kaposi's sarcoma herpesvirus and the cytoplasmic Tax protein of human T-cell leukemia virus type 1 associate directly with IKKγ (NEMO), leading to constitutive NF-κB activation [63,64].

Until now, most research in the *Theileria* field has been hypothesis-driven, focusing on candidate pathways and kinases likely to impact apoptosis, proliferation, or cell motility. It is striking to consider how many of the proteins targeted by *Theileria* are of central importance for other diseases. The transformed phenotype induced by *Theileria* is reversible, and with this in mind comparative proteomics, phosphoproteomics, transcriptomics and epigenomic studies performed in this system can be expected to provide valuable new insights into the deregulation of cellular control mechanisms involved not only in theileriosis but also in other proliferative diseases, especially those involving the immune system. The details of a first analysis of host cell gene expression networks in *Theileria* infection has recently been published, and novel candidate genes potentially involved in *Theileria*-induced transformation, such as the pro-cancer genes MMP13 (matrix metallopeptidase 13), NDRG1 (N-myc downstream regulated), ICAM1 (intercellular adhesion molecule-1) and SPARC (secreted protein acidic and rich in cysteine), have been tentatively proposed [6]. Such studies might also open new lines of research for other host–pathogen interactions.

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