HHS Public Access

Author manuscript

Curr Opin Microbiol. Author manuscript; available in PMC 2021 April 04.

Published in final edited form as:

Curr Opin Microbiol. 2020 December ; 58: 99–105. doi:10.1016/j.mib.2020.09.009.

The RESTRICTION checkpoint: A window of opportunity governing developmental transitions in *Toxoplasma gondii*.

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Abstract

The life cycle of *Toxoplasma gondii* is characterized by active replication alternating with periods of rest. Encysted dormant sporozoites and bradyzoites initiate active replication as tachyzoites and merozoites. Here we explore the role of the cell cycle with a focus on the canonical G_1 RESTRICTION checkpoint (R-point) as the integrator governing developmental decisions in *T. gondii*. This surveillance mechanism which licenses replication, creates a window of opportunity in G_1 for cellular reorganization in the execution of developmental transitions. We also explore the unique status of the bradyzoite, the only life cycle stage executing both a forward (entry into the sexual cycle) and reverse (recrudescence) developmental transitions as a multipotent cell. These opposing decisions are executed through the common machinery of the RESTRICTION checkpoint.

Keywords

Toxoplasma gondii; growth; development; cell cycle; checkpoint; metabolism

INTRODUCTION

The life cycle of the apicomplexan parasite *Toxoplasma gondii* is comprised of alternating periods of replication and rest, with developmental decisions governing the transitions (Fig. 1). Upon infection of an intermediate host, resting sporozoites convert into rapidly dividing tachyzoites [1,2]. The rapid expansion of progeny triggers a potent immune response and changes in the nutritional environment [3]. This causes tachyzoites to slow their division converting into largely dormant bradyzoites [3]. After transmission to the definitive feline host, bradyzoites initiate the replicative stage of merogony which is followed by

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morphological transitions to generate gametocytes [4,5]. Gametocytes fuse and form unsporulated oocysts [6]. Meiosis and limited rounds of replication convert each zygote into a mature oocyst with eight non-replicative sporozoites [5]. Bradyzoite infection of intermediate hosts results in conversion to tachyzoites which can also occur during tissue cyst reactivation within a host [3]. Rapid division of tachyzoites is responsible for the symptomatic stages of infection, while resting bradyzoites associate with the silent chronic infection [3]. Of note, only encysted resting stages (bradyzoites and sporozoites) are capable of transmission between intermediate and definitive hosts [1,7], which involves re-initiation of the active growth.

Our understanding of regulation of the stage conversions has been largely limited to studies of the unidirectional tachyzoite to bradyzoite development [8]. Most of the insights emerge from *in vitro* systems using diverse triggers [8]. The role of extrinsic stressors is supported by *in vivo* observations which strongly implicate infection-induced inflammation as a major trigger of differentiation [9]. Accumulation of such extrinsic factors as IFN and other cytokines [10,11] creates an unfavorable host environment marked by toxic reactive oxygen and nitrogen species [12,13]. Furthermore, the inflammatory state is associated with metabolic changes impacting key building blocks and affecting cell energy metabolism [14]. Due to *T. gondii* auxotrophies, limitation in arginine and tryptophan arrests parasite biosynthetic activity [15]. A new cellular environment in the host represses parasite replication promoting a resting stage that had long been considered as bradyzoite dormancy [16]. The microenvironment created by immune effectors is central to the maintenance of the parasite rest *in vivo*, as the loss of this control significantly increases reactivation and a return to active growth [3].

The bradyzoite is the only life cycle stage that can execute both a forward step to merozoites and the reverse step to tachyzoites (Fig. 1). The contribution of lipid metabolism for the forward step has been elegantly demonstrated by the Knoll laboratory as involving the sensing of accumulated unsaturated fatty acids (linoleic acid) [17]. This anomaly specific to feline intestinal physiology explains the highly restricted *T. gondii* host range for the sexual cycle. Nevertheless, despite of the clinical importance of the rest-growth transitions, we know very little about the signals and how the commands are executed.

How are diverse extrinsic stimuli governing parasite developmental transitions are processed within the parasite? The machinery to promote these changes is likely integrated into the basic physiology of the parasite that governs the decision to grow or not. At the heart of this decision is the canonical cell cycle RESTRICTION (or R-point) checkpoint. Here we present arguments pointing to the crucial role of the R-point surveillance mechanism in determining *T. gondii* developmental fate. In addition, we propose that the bradyzoite functionally resembles a multipotent cell whose fate is determined by the specific factors/signals processed at the RESTRICTION checkpoint.

RESTRICTION checkpoint balances replication and differentiation in *T. gondii*

Cell division is regulated by the set of surveillance mechanisms called checkpoints to ensure orderly progression through the cell cycle [18]. Located in the G_1 phase the RESTRICTION checkpoint is a molecular switch that controls the binary decision to enter or not enter the

DNA replication phase [19], consequently, to divide or delay division. This universal mechanism mediates cell adaptation to the changing environment by balancing replication and rest. Originally viewed as a G_1 stop, the R-point was later re-evaluated as a cell cycle period or "window of opportunity" that is critically needed to introduce intracellular changes [20]. Undoubtedly, the R-point functions in apicomplexan parasites, including *T. gondii*. It has been shown that encysted *T. gondii* stages, sporozoites and bradyzoites, are primarily a G_1 form of the parasite, suggesting retention within the window of opportunity [21].

Depending on the conditions, R-point passage may be swift or prolonged, which translates into a growth or rest, respectively. We propose that the R-point mechanism supports the gradual change of the *T. gondii* tachyzoite into the bradyzoite (Fig. 2A). Rapidly dividing tachyzoites have a short G_1 phase, indicative of the uninterrupted R-point passage. Upon bradyzoite differentiation the proportion of the G_1 parasites increases reaching nearly 100% G_1 parasites in the late stages [21–23]. This phenomenon can be explained by dramatic temporal reorganization of the parasite cell cycle recently verified by single-cell RNA sequencing approach [23]. At each division cycle, the G_1 phase of differentiating bradyzoites disproportionally extends with little or no change in the S-phase, mitosis or budding (Fig. 2A). In contrast to a common view, persistent differentiation signal does not trigger the exit from the cell cycle (G_0), but rather intervenes with the R-point passage making it increasingly difficult to enter the S-phase, thereby blocking commitment to replication. This mechanism explains why in the late stages, larger proportions of bradyzoites are detained within the widening window of opportunity that progressively extends the cell cycle duration (Fig. 2A). We recently demonstrated that even at the late stage of development, bradyzoites do divide within the tissue cysts *in vivo* [22]. Therefore, despite increasing difficulty to progress through the G_1 phase, once all the checkpoint conditions are met, licensed bradyzoites pass the R-point and complete a division cycle.

Although the R-point is a binary switch, the length of the window of opportunity determines the degree of changes introduced at each division cycle [24]. For example, bradyzoite maturation can be modeled as sequential, increasingly difficult passages through the R-point (Fig 2A). The tachyzoite's window of opportunity is too short to introduce developmental changes. Differentiation signals initiate re-programming in G_1 executed at the epigenetic, transcriptional and translational levels (Fig. 2B) [23,25–28]. Implementation of the developmental reprogramming has been recently demonstrated in the *in vitro* studies of transcriptional regulators. Among these are TgBDF1, several AP2 factors and MORC1 complex which either promote or inhibit the developmental transitions, or dysregulate these processes [25,28–32]. Active reprogramming establishes a new normal that promotes further epigenetic changes to assure the new state is sustained. Gene expression changes coupled with post-translational modifications culminate in adaptive changes of parasite growth and physiology most clearly seen with regard to energy metabolism (Fig. 2C) [15] [33–35]. The *in vitro* and more recently *in vivo* bradyzoite (Patwardhan and Sinai, unpublished) studies reveal altered mitochondrial activity/morphology [36], and changes in the glucose storage, amylopectin granules [37,38]. Additionally, changes in amino acid transport/synthesis are also observed as is likely the case for other classes of metabolites [15]. Cumulative external signals from the host and altered metabolism of the developing bradyzoite continue to apply pressure and make the R-point passage more challenging. Consequently, lengthening the

window of opportunity allows extensive reprogramming and further changes in metabolism. Under such conditions, the rounds of the parasite division create a feedback loop building a deeper stage of the bradyzoite differentiation. Evidence from diverse experimental systems show that developmental transitions are probabilistically driven by accumulation of small changes, until a threshold is reached [39]. As more parasites reach the threshold, the population shifts, an event we recognize as the specific stage conversion.

RESTRICTION checkpoint regulation in *T. gondii*

The RESTRICTION checkpoint is operated by a set of factors that connect environmental signals to the biosynthetic pathways [40]. In conventional eukaryotes, the core R-point network is composed of the CDK4/6 family kinases, D-type cyclins and the specific CDK effector, Retinoblastoma protein, that coordinate gene expression by activation of the e2F transcription factor [40]. None of these canonical R-point regulators were found in apicomplexans, including *T. gondii*, suggesting that apicomplexan R-point is regulated by alternative network [41]. We recently demonstrated that *T. gondii* tachyzoites deficient in either CDK-related kinase TgCrk2 or P-type cyclin struggle to complete G_1 [42]. Stable interaction of TgCrk2 kinase with TgPHO80 cyclin makes this complex the most likely candidate for the R-point regulator in *T. gondii*. A few studies indicate that the TgCrk2/P-cyclins network may function as a universal regulator of the RESTRICTION checkpoint in apicomplexan parasites and other protozoa. Orthologs of TgCrk2 and P-cyclins are encoded in all Apicomplexa and were also found in kinetoplastid genomes [42–45]. The TgCrk2 related kinase CRK1 and P-cyclins regulate the G_1/S transition and cytoskeletal morphogenesis in *Trypanosoma brucei* [43,45,46], while studies of *Plasmodium berghei* development demonstrated a critical role for P-cyclin CYC3 in oocyst sporulation in mosquito stages [44]. Interestingly, TgCrk2 and P-cyclins are distantly related the PHO (phosphate) network found in yeast and plants [43,46–49], but are absent in animals [41,42,44]. In yeast, PHO network regulates various G_1 functions, including ion-sensing pathways, nutrient uptake and carbon utilization [50,51]. Moreover, PHO complexes control the passage through the yeast START checkpoint analogous to the R-point in higher eukaryotes [52]. Similar function of the yeast PHO and *T. gondii* G_1 network suggests origination from the same inherited requirements to regulate replication in response environmental changes.

Cyst heterogeneity and developmental fate of the *Toxoplasma* bradyzoite

In mammals, the RESTRICTION checkpoint plays a key role in maintenance and fate commitment of the pluripotent cells, suggesting ancestral functions of the R-point in control population diversity [24,53]. There are clear parallels between differentiation of the mammalian stem cells and differentiation in *T. gondii*, particularly as relates to bradyzoites (Fig. 3). The multipotency of the bradyzoite allows the parasite to remain in its resting state, convert to a tachyzoite or initiate merogony (Fig. 1 and 3). Developmental plasticity of the bradyzoite responding to its extrinsic environment by integrating intrinsic signals into decisions that alter the developmental fate of its immediate progeny, mirrors mammalian stem cell behavior. In our view these decisions are intimately tied to the G_1 function. At the RESTRICTION checkpoint, bradyzoites integrate extrinsic (immune environment and metabolites) and intrinsic cues (parasite metabolic state) and execute the developmental

programs in a stepwise fashion to reach thresholds associated with the transition. Specificity of the signals drives specific gene expression leading a functional shift in the life cycle stage.

One of the outstanding characteristics of the bradyzoite stage is a remarkable diversity of the intracyst population [22,23]. It is tempting to suggest that cyst heterogeneity facilitates the developmental choice of individual bradyzoites. Early bradyzoites may have higher chance to reinitiate tachyzoite cycle while mature bradyzoites may efficiently converting into merozoite stage. The maintenance of heterogeneity is likely under the cell cycle control. Bradyzoite diversity is probably the result of the cell cycle changes defined by the duration of the window of opportunity creating both cell cycle and physiological diversity. Although the average cyst consists of primarily the G_1 bradyzoites, metabolic state of the parasites passing thought the window of opportunity of the first division cycles will be very different than the state of the parasites progressing through the G_1 phase of the later division cycles. Indeed, our recent work revealed that individual bradyzoites within tissue cysts, while being genetically clonal are remarkably heterogenous with regard to their physiology [22,23]. This is seen most clearly in the loss of replicative synchrony, diversity in mitochondrial activity/morphology (Patwardhan and Sinai, unpublished) as well as in the levels, distribution and utilization of the amylopectin granules ([38,54], Murphy, Gentry and Sinai, unpublished). As we model transitions in the *T. gondii* life cycle, quantification of the physiological parameters coupled with detailed “omic” studies at the single cell level are going to be crucial to dissecting how the window of opportunity is harnessed to execute developmental transitions.

In conclusion, confirmation of the R-point’s role in *T. gondii* developmental will require elucidation of how the stage-specific stimuli are integrated. While the immune and metabolic environment for each transition is different, the corresponding signals converge at and recognized by the R-point machinery. Identification of stimuli, pathways and factors regulating the clinically relevant bradyzoite reactivation will be of a particular importance. Given the central role of the R-point in the growth-rest decision, the checkpoint dissection is likely to reveal targets for pharmacological intervention that may raise R-point barrier thereby block reactivation. This and fundamental discoveries about how environmental and metabolic cues are integrated in the basic decisions governing the *Toxoplasma* growth offers a new perspective for ongoing investigations into a fascinating parasite.

AKNOWLEDGMENTS

The work was supported by grants awarded to ESS R01AI141467 and APS R01AI145335.

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HIGHLIGHTS

- The developmental fate of *Toxoplasma gondii* is decided at the G_1 RESTRICTION checkpoint (R-point).
- The G_1 elongation creates a window of opportunity to integrate cues into developmental changes.
- Specific host signals direct development of the multipotent bradyzoites at the R-point.
- The inherent heterogeneity of individual bradyzoites is a direct consequence of cell cycle reconstruction.

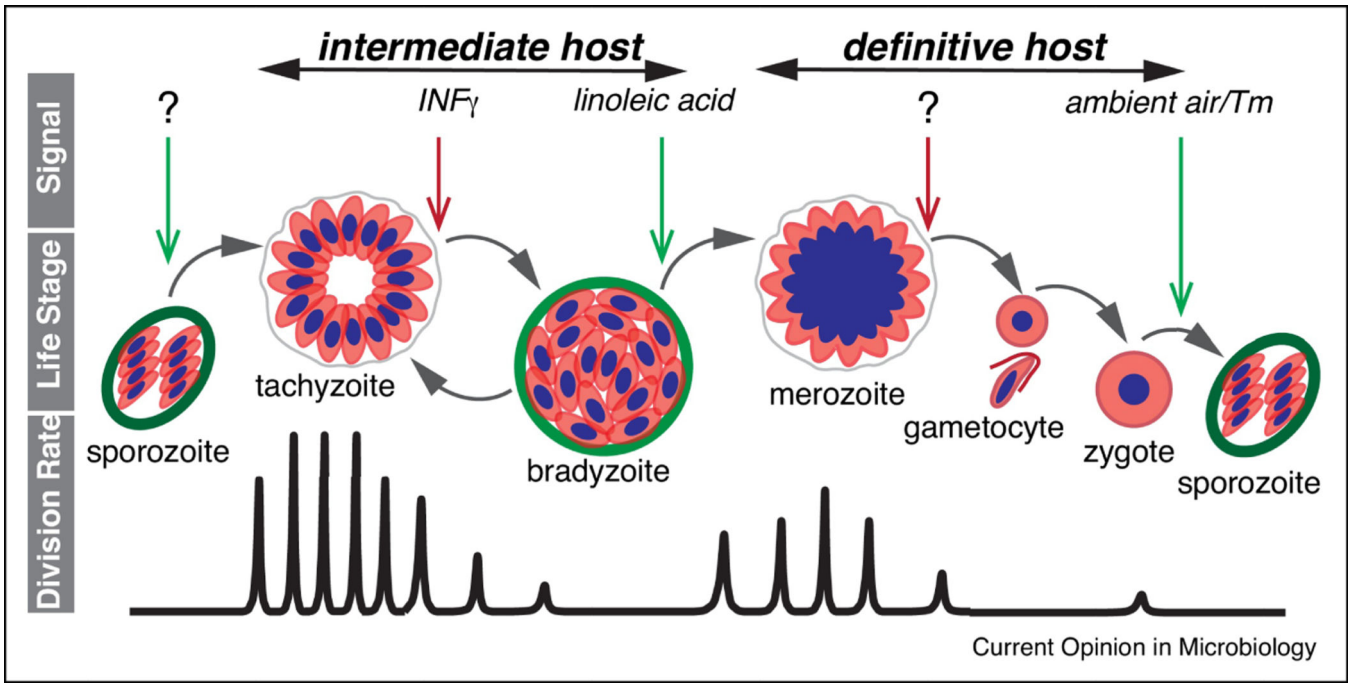

Figure 1. Major growth-rest transitions in the *T. gondii* life cycle.

Diagram highlights correlation between extrinsic signals from the host, parasite life stages and replication rates. Transitions between parasite life stages are driven by the host-specific signals, many of which are still unknown. Green arrows indicate signals to enhance parasite division, and the red arrows specify signals to repress division. Alternating *T. gondii* life stages are indicated in the middle cartoon. Bottom graph visualizes the frequency of the cell division as parasite progresses through the life cycle. First infection of intermediate and definitive host is caused by the resting *T. gondii* forms, sporozoite and bradyzoite respectively. Each host supports at least one replicative and one resting form of the parasite. Replication in different hosts follow different mechanisms. Tachyzoite employs binary division, endodyogeny to amplify in intermediate hosts, while merozoites divide by multinuclear mechanism, endopolygeny, in definitive feline host. Note that besides the self-renewal, the bradyzoite is the only *T. gondii* stage capable of forward (merozoite) and reverse (tachyzoite) development.

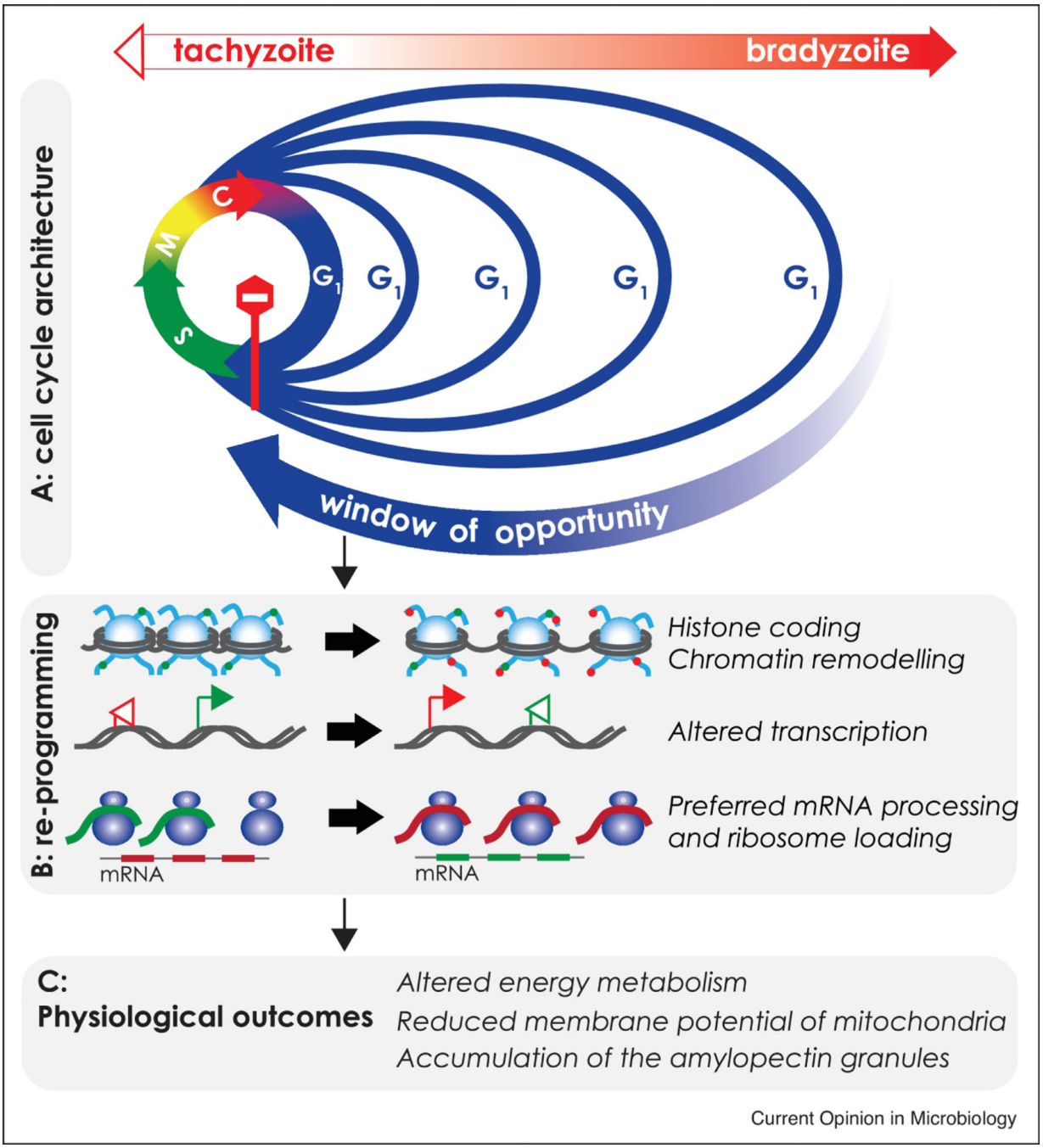

Figure 2. The RESTRICTION checkpoint controls developmental transitions in *T. gondii*.

Diagram illustrates a tachyzoite-to-bradyzoite conversion**. A.** Reorganization of the cell cycle upon bradyzoite development. Color arrows circle represents cell cycle phases. Blue – Gap 1 (G_1) phase, devoted to cell growth and preparation for cell division. Green – a Synthesis (S)-phase, devoted to DNA replication. Yellow – Mitosis (M) phase, during which the sister chromatids are segregated on bipolar spindle. Red – cytokinesis (C), is the last stage of mitosis where the mother cell is resolved into two daughter cells. Stop sign indicates the threshold of the RESTRICTION checkpoint where external signals govern the entry into

division cycle. Rapidly dividing tachyzoite has a short G_1 period (blue arrow). Unfavorable bradyzoite differentiation conditions repress cell division by disproportional increase of the G_1 phase (blue line). This change creates a window of opportunity much needed for cell reprogramming. **B.** Major reprogramming events taking place in G_1 phase of the developing bradyzoites. Window of opportunity permits regional relaxation of the chromatin and changes in histone coding, which in turn activates transcription of the previously silent genes. Transcripts of the bradyzoite-specific genes receive preferential processing. Green colored modifications are tachyzoite specific and red colored are bradyzoite specific. **C.** Parasite reprogramming during window of opportunity culminates in indicated metabolic changes. New metabolic status of the parasite becomes an internal signal to maintain the current developmental pathway.

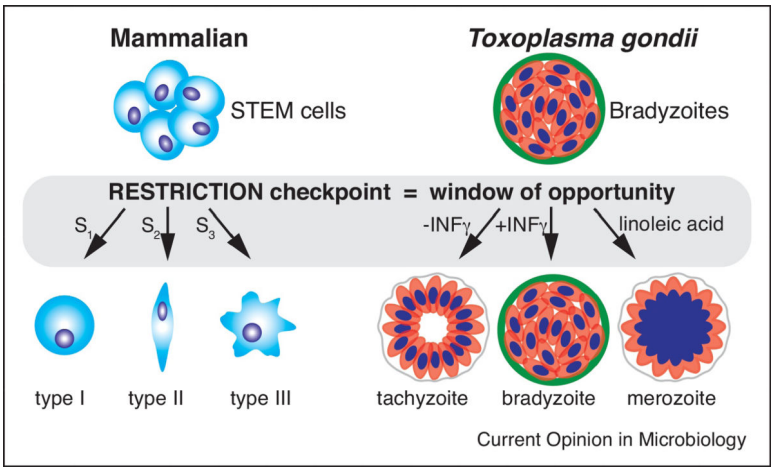

Figure 3. Bradyzoite is the only multipotent *T. gondii* stage.

Schematics on the left (blue colored cells) represents ability of the mammalian STEM cells to convert into several type of cells. Different signals (S) trigger different developmental pathways (cell type). Similarly, alternative external stimuli direct development of the *T. gondii* bradyzoites (red colored cells) toward different fates. Bradyzoites recrudesce into tachyzoites when IFN**γ** is depleted, while excess of the linoleic acid drives forward development into merozoites. In both models, signals are converted into a specific developmental program during window of opportunity in the G_1 phase (grey area).