



# Critical functions of the polyamine putrescine for proliferation and viability of *Leishmania donovani* parasites

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## Abstract

Polyamines are metabolites that play important roles in rapidly proliferating cells, and recent studies have highlighted their critical nature in *Leishmania* parasites. However, little is known about the function of polyamines in parasites. To address this question, we assessed the effect of polyamine depletion in *Leishmania donovani* mutants lacking ornithine decarboxylase ( $\Delta odc$ ) or spermidine synthase ( $\Delta spdsyn$ ). Intracellular putrescine levels depleted rapidly in  $\Delta odc$  mutants and accumulated in  $\Delta spdsyn$  mutants, while spermidine levels were maintained at low but stable levels in both cell lines. Putrescine depletion in the  $\Delta odc$  mutants led to cell rounding, immediate cessation of proliferation, and loss of viability, while putrescine-rich  $\Delta spdsyn$  mutants displayed an intermediate proliferation phenotype and were able to arrest in a quiescent-like state for 6 weeks. Supplementation of  $\Delta odc$  mutants with spermidine had little effect on cell proliferation and morphology but enabled parasites to persist for 14 weeks. Thus, putrescine is not only essential as precursor for spermidine formation but also critical for parasite proliferation, morphology, and viability.

**Keywords** *Leishmania* · Polyamines · Putrescine · Gene deletion mutants · Proliferation · Viability

## Abbreviations

|          |                                  |
|----------|----------------------------------|
| ADOMETDC | Adenosylmethionine decarboxylase |
| ARG      | Arginase                         |
| DFMO     | Difluoromethylornithine          |
| DHS      | Deoxyhypusine synthase           |
| DOOH     | Deoxyhypusine hydroxylase        |
| ODC      | Ornithine decarboxylase          |
| PBS      | Phosphate buffered saline        |
| SPDSYN   | Spermidine synthase              |
| SPMSYN   | Spermine synthase                |

|      |                                  |
|------|----------------------------------|
| TCA  | Trichloroacetic acid             |
| TRYS | Trypanothione synthetase–amidase |

## Introduction

Parasites of the genus *Leishmania* cause a variety of devastating diseases in humans and domestic animals worldwide. The spectrum of leishmaniasis ranges from cutaneous ulcerative lesions to fatal visceralizing infections, and affects annually an estimated 12 million people worldwide (Kedzierski 2011). Among diseases of parasitic origin, visceral leishmaniasis is the second leading cause of mortality in humans (Alvar et al. 2012; Bern et al. 2008; Kaye and Scott 2011). The parasite exhibits a digenetic life cycle in which the extracellular promastigotes reside in the gut of the sand fly vector, whereas the intracellular amastigotes inhabit the phagolysosomes of macrophages in the infected mammalian host. Due to the absence of effective vaccines, chemotherapy offers the only avenue of defense against leishmaniasis (Kaye and Scott 2011; Kedzierski et al. 2009; Muller 2007). However, the currently available small arsenal of drugs used to treat leishmaniasis is far from ideal due to a lack of selectivity and emergence of drug resistance (Croft

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et al. 2006; Mishra et al. 2007; Ponte-Sucre et al. 2017; Singh et al. 2012). Thus, the need for a better understanding of parasite biology to develop new therapeutic strategies is urgent.

One pathway that has already been validated as a therapeutic target in the related pathogen, *Trypanosoma brucei gambiense*, is that for polyamine biosynthesis (Babokhov et al. 2013; Bacchi and McCann 1987; Burri and Brun 2003; Docampo and Moreno 2003; Fairlamb 2003). D,L- $\alpha$ -Difluoromethylornithine (DFMO) is a suicide inhibitor of ornithine decarboxylase (ODC), the enzyme that catalyzes putrescine biosynthesis, and shows remarkable therapeutic efficacy in treating African sleeping sickness caused by *T. brucei gambiense* (Babokhov et al. 2013; Bacchi and McCann 1987; Burri and Brun 2003; Docampo and Moreno 2003; Fairlamb 2003). DFMO is also active against other protozoan parasites in vitro, including *Plasmodia* and *Giardia* (Bitonti et al. 1987; Gillin et al. 1984) and is effective against *Leishmania* in vitro and in murine and hamster infectivity models (Boitz et al. 2009; Gradoni et al. 1989; Kaur et al. 1986; Mukhopadhyay and Madhubala 1993; Olenyik et al. 2011).

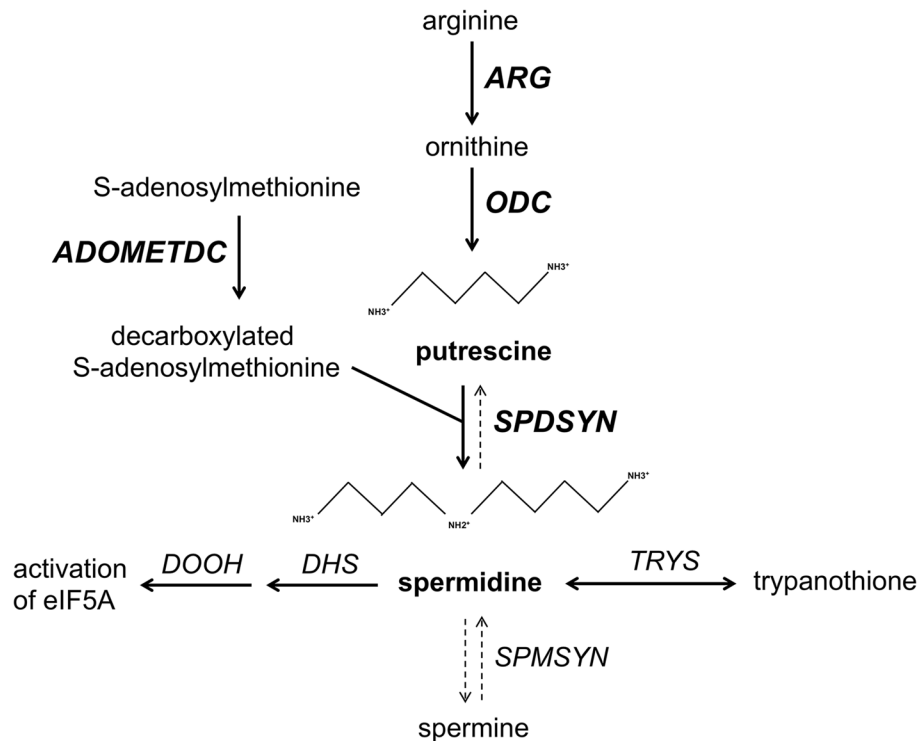
The polyamines putrescine, spermidine, and spermine are ubiquitous and essential cations that play critical roles in proliferation and survival, although their exact functions are still unclear (Igarashi and Kashiwagi 2010; Lenis et al. 2017; Miller-Fleming et al. 2015; Minois et al. 2011; Pegg 2016). Whether the three polyamines have different or overlapping functions and whether all three polyamines are essential remains unknown (Bachrach et al. 2001; Igarashi and Kashiwagi 2010). These analyses are particularly difficult due to the inter-conversion pathways that exist in mammalian cells (Seiler 2004; Seiler et al. 1981; Wang and Casero 2006). As putrescine levels are typically low in mammalian cells, this polyamine is often considered to be merely a precursor for spermidine and spermine formation (Battaglia et al. 2014; Casero and Marton 2007; Kahana 2018; Murray-Stewart et al. 2016; Tavladoraki et al. 2012). It is intriguing that while spermidine and spermine predominate in metazoans, putrescine and spermidine are more abundant in rapidly proliferating prokaryotes and unicellular eukaryotes (Igarashi and Kashiwagi 2010). In contrast to the plethora of studies on the functions of polyamines in mammalian cells, little is known about the functions of polyamines in protozoan parasites, although recent studies have highlighted the importance of the polyamine biosynthetic pathway as a potential therapeutic target (Birkholtz et al. 2011; Bisceglia et al. 2018; Heby et al. 2003, 2007; Ilari et al. 2015, 2017; Phillips 2018; Roberts and Ullman 2017).

The polyamine biosynthetic pathway in *Leishmania* consists of four enzymes: arginase (ARG), ODC, spermidine synthase (SPDSYN), and *S*-adenosylmethionine decarboxylase (ADOMETDC) (Fig. 1). ARG, the first and committed

step in polyamine biosynthesis, converts arginine to ornithine, which is subsequently metabolized to the diamine putrescine by the catalytic action of ODC. SPDSYN then generates spermidine via the addition of an aminopropyl group donated from decarboxylated *S*-adenosylmethionine. Spermine, a prevalent polyamine of higher eukaryotes, is neither synthesized nor utilized by *Leishmania* (Jiang et al. 1999) and no spermine synthase homolog can be found in *Leishmania* genomes (El-Sayed et al. 2005). Unique to trypanosomatids, which include *T. brucei*, *T. cruzi*, and *Leishmania* spp., is the conjugation of spermidine and glutathione to form trypanothione. This metabolite is essential to combat oxidative stress in parasites (Colotti and Ilari 2011; Ilari et al. 2017; Krauth-Siegel et al. 2003, 2007; Krauth-Siegel and Comini 2008; Krauth-Siegel and Inhoff 2003; Manta et al. 2013, 2018). In *Leishmania*, the enzyme trypanothione synthetase–amidase (TRYSA) catalyzes the reversible formation of trypanothione (Fyfe et al. 2008). Spermidine is also used for the hypusination and activation of eukaryotic initiation factor 5A (eIF5A). The enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOOH) are responsible for this reaction that has been found to be essential in *Leishmania* (Chawla et al. 2010, 2012) and the mammalian host (Park and Wolff 2018).

ARG, ODC, SPDSYN, and ADOMETDC have all been validated as indispensable for the promastigote form of *Leishmania donovani*, as gene knockouts of each enzyme confer polyamine auxotrophy to the mutants, which can only be grown in the presence of appropriate polyamine supplementation (Jiang et al. 1999; Roberts 2013; Roberts et al. 2001, 2002, 2004). Studies with  $\Delta arg$  knockout mutants in several *Leishmania* species established that the sole essential role of ornithine is as a precursor for polyamine formation (Boitz et al. 2016; da Silva and Floeter-Winter 2014; da Silva et al. 2012; Reguera et al. 2009; Roberts et al. 2004). In contrast, the role of putrescine is more controversial. Although spermidine is the final polyamine of the pathway in *Leishmania*, and some studies suggest that spermidine is indeed the only essential polyamine (Jiang et al. 1999; Reguera et al. 2009), a more recent study shows that putrescine is essential beyond its role as a precursor for spermidine formation (Boitz et al. 2016).

Murine infectivity studies have established an important role for parasite ARG in several cutaneous *Leishmania* species, *L. mexicana*, *L. major*, and *L. amazonensis* (da Silva et al. 2012; Gaur et al. 2007; Muleme et al. 2009; Reguera et al. 2009), and the visceralizing species *L. donovani* (Boitz et al. 2016). The  $\Delta arg$  knockout mutants in these strains exhibit lower infectivity levels than the corresponding wild-type parasites, although they are still able to establish infections (Boitz et al. 2016; da Silva et al. 2012; Gaur et al. 2007; Muleme et al. 2009; Reguera et al. 2009). In contrast,  $\Delta odc$  and  $\Delta spdsyn$  gene deletion mutants in *L. donovani* elicit profound



**Fig. 1** The polyamine biosynthetic pathway. The polyamine biosynthetic pathway in *Leishmania* parasites is outlined in bold arrows. The linear conversion of arginine to ornithine, putrescine, and spermidine is catalyzed by arginase (ARG), ornithine decarboxylase (ODC), and spermidine synthase (SPDSYN), respectively. The enzyme *S*-adenosylmethionine decarboxylase (ADOMETDC) forms decarboxylated *S*-adenosylmethionine, the aminopropyl-group donor for the formation of spermidine. The two polyamines found in *Leishmania* parasites, putrescine and spermidine, are bolded. Unique to trypano-

matids is the formation of trypanothione; in *Leishmania* this reversible reaction is catalyzed by trypanothione synthetase–amidase (TRYS). The modification and activation of eukaryotic initiation factor 5A (eIF5A) by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOOH) occurs in both *Leishmania* parasites and the human host. Denoted in dashed arrows are the spermine synthase (SPMSYN) reaction and the simplified back-conversion pathway that occur in the mammalian host but are not present in *Leishmania* parasites

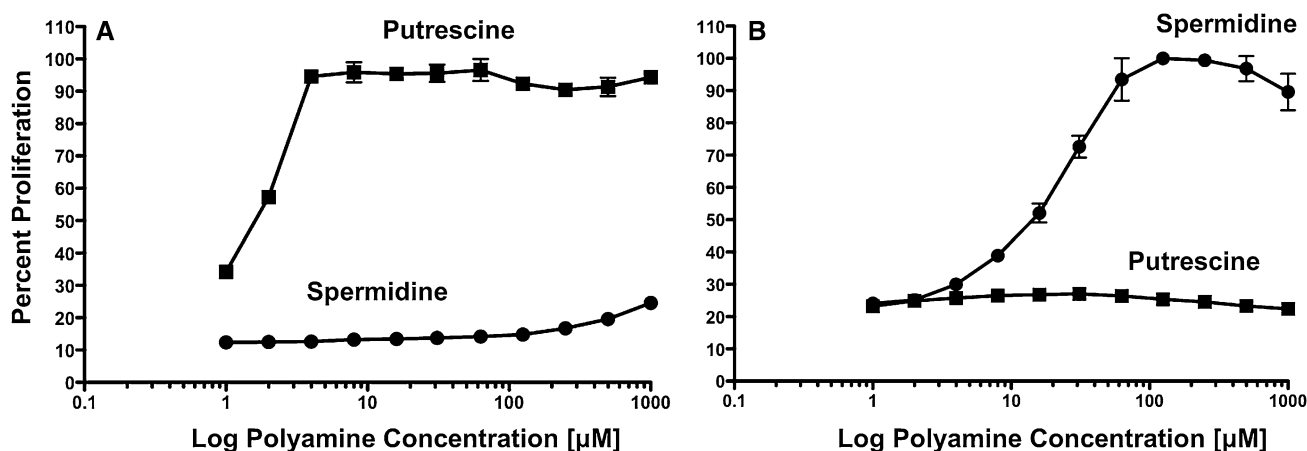
reductions in parasite loads in infected mouse organs, with the  $\Delta odc$  deletion having the most striking effect (Boitz et al. 2009; Gilroy et al. 2011). These studies established the importance of polyamine biosynthesis in intracellular *Leishmania* parasites.

In this study, we have examined the consequences of polyamine depletion in *L. donovani*  $\Delta odc$  and  $\Delta spdsyn$  gene deletion mutants to better understand the functions of polyamines in *Leishmania*. Putrescine was not only essential as a precursor for spermidine formation but also critical for parasite proliferation, morphology, and viability. Furthermore, while the absence of both polyamines caused imminent cell death, the presence of either putrescine or spermidine alone, allowed parasites to arrest in a quiescent-like state for several weeks.

## Materials and methods

### Materials

Dulbecco's Modified Eagle's Medium, chicken serum, and phleomycin were procured from Thermo Fisher Scientific (Waltham, MA, USA). Resazurin, putrescine, and spermidine were purchased from VWR International (Radnor, PA, USA). Dansyl chloride was obtained from Sigma-Aldrich (Burlington, MA, USA), and the Muse Count and Viability reagent was purchased from Millipore (Burlington, MA, USA).



**Fig. 2** Putrescine and spermidine concentrations required to rescue mutant parasites. The  $\Delta odc$  (a) and  $\Delta spdsyn$  (b) promastigotes were incubated in serial dilutions of 1000  $\mu\text{M}$  putrescine (black squares) or 1000  $\mu\text{M}$  spermidine (black circles). Viability was evaluated after

5 days by measuring the conversion of resazurin to resorufin. Data are from two biological replicates ( $n=2$ ) with error bars representing standard deviations. The experiment was repeated two more times with essentially the same outcome

## Cell culture and cell lines

All genetically manipulated parasites were derived from the wild-type LdBob strain of *L. donovani* (Goyard et al. 2003) that was originally obtained from Dr. Stephen M. Beverley (Washington University, St. Louis, MO, USA). The  $\Delta odc$  and  $\Delta spdsyn$  mutants were previously generated by targeted gene replacement techniques (Boitz et al. 2009; Gilroy et al. 2011). Promastigote parasites were incubated at 27 °C in a completely defined Dulbecco's Modified Eagle-based culture medium especially designed for the cultivation of *Leishmania* promastigotes, where fetal bovine serum was replaced with chicken serum to avoid polyamine oxidase-mediated toxicity (DME-L CS) (Iovannisci and Ullman 1983; Kaur et al. 1986; Roberts et al. 2001). The  $\Delta spdsyn$  cell line was maintained in 100  $\mu\text{M}$  spermidine and the  $\Delta odc$  cell line was routinely grown in the presence of 100  $\mu\text{M}$  putrescine, unless otherwise specified. Prior to performing the experiments described in this paper, the phenotype of the  $\Delta odc$  and  $\Delta spdsyn$  gene deletion mutants was verified by western blot analysis. Blots were probed with polyclonal rabbit antibodies raised against ODC and SPDSYN (Jiang et al. 1999; Roberts et al. 2001) and tubulin as a loading control, and the absence of ODC in the  $\Delta odc$  cell lines and SPDSYN in the  $\Delta spdsyn$  parasites was confirmed (Supplemental Fig. 1).

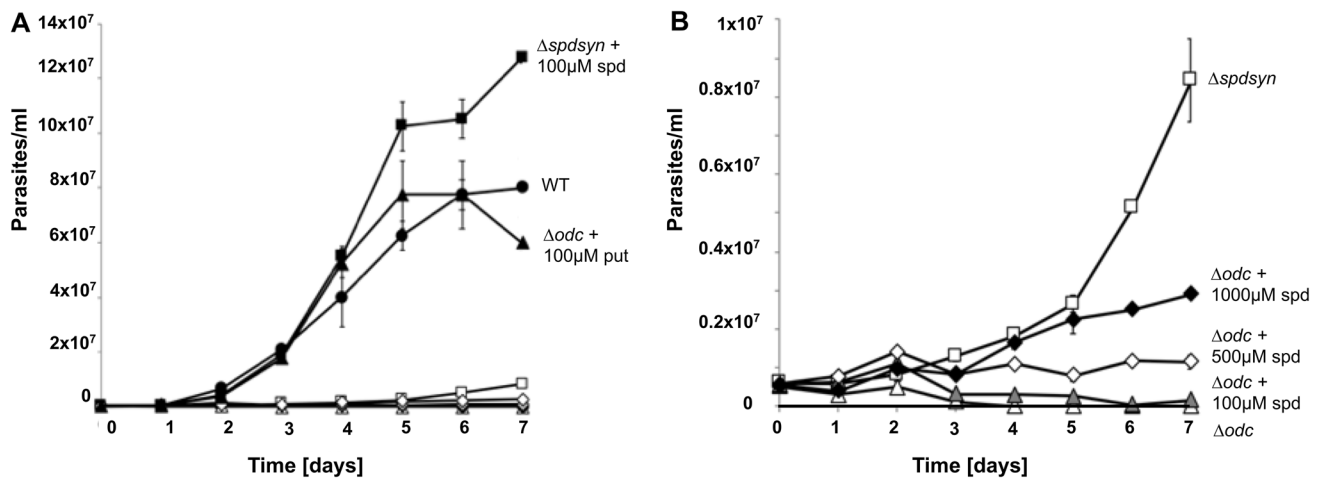
## Assessment of growth phenotypes

Wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were allowed to reach stationary phase in media supplemented with no polyamines (wild-type), with 100  $\mu\text{M}$  putrescine ( $\Delta odc$ ), or with 100  $\mu\text{M}$  spermidine ( $\Delta spdsyn$ ). Parasites were then harvested and washed three times in phosphate buffered saline (PBS),

counted on a hemocytometer and seeded at defined cell numbers for all growth assays. To determine 50% effective concentration ( $EC_{50}$ ) values and concentrations of putrescine and spermidine required for optimal growth,  $\Delta odc$  and  $\Delta spdsyn$  parasites were seeded in a volume of 100  $\mu\text{l}$  in 96-well plates at a density of  $5 \times 10^4/100 \mu\text{l}$  in serial dilutions of 1000  $\mu\text{M}$  putrescine or 1000  $\mu\text{M}$  spermidine. After 5 days, 15  $\mu\text{l}$  of 250  $\mu\text{M}$  resazurin was added to each well, and plates were incubated for an additional 4 h. To assess cellular proliferation, conversion of resazurin to resorufin was evaluated on a BioTek Synergy plate reader by monitoring fluorescence ( $579_{Ex}/584_{Em}$ ). Graphs were prepared using GraphPad Prism version 6.0f for Mac. Data shown in Fig. 2 are from biological duplicates ( $n=2$ ). The experiment was repeated two more times with essentially the same outcome. To determine daily parasite growth rates, wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were seeded in a volume of 10 ml at a density of  $5 \times 10^5/\text{ml}$  and counted in a hemocytometer daily for 7 days. The  $\Delta odc$  and  $\Delta spdsyn$  parasites were incubated in media containing no polyamines, 100  $\mu\text{M}$  putrescine, 100  $\mu\text{M}$ , 500  $\mu\text{M}$ , or 1000  $\mu\text{M}$  spermidine as indicated. Graphs were prepared using Microsoft Excel. Data displayed in Fig. 3 are from biological duplicates ( $n=2$ ). The experiment was repeated two more times with essentially the same outcome.

## Polyamine pool analysis

Wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were allowed to reach stationary phase in media supplemented with no polyamines (wild-type), with 100  $\mu\text{M}$  putrescine ( $\Delta odc$ ), or with 100  $\mu\text{M}$  spermidine ( $\Delta spdsyn$ ). Parasites were then harvested and washed three times in PBS. Wild-type parasites were



**Fig. 3** Proliferation of wild-type and mutant parasites in supplemented or un-supplemented media. Parasites were seeded at  $5 \times 10^5$  parasites/ml and proliferation was evaluated daily by counting parasite numbers on a hemocytometer. **a** Depicts growth of all cell lines, while **b** focuses on growth of starved parasites to allow a better comparison of low cellular proliferation rates. Wild-type parasites were incubated in un-supplemented media (black circles),  $\Delta odc$  parasites were incubated in un-supplemented media (white triangles) or sup-

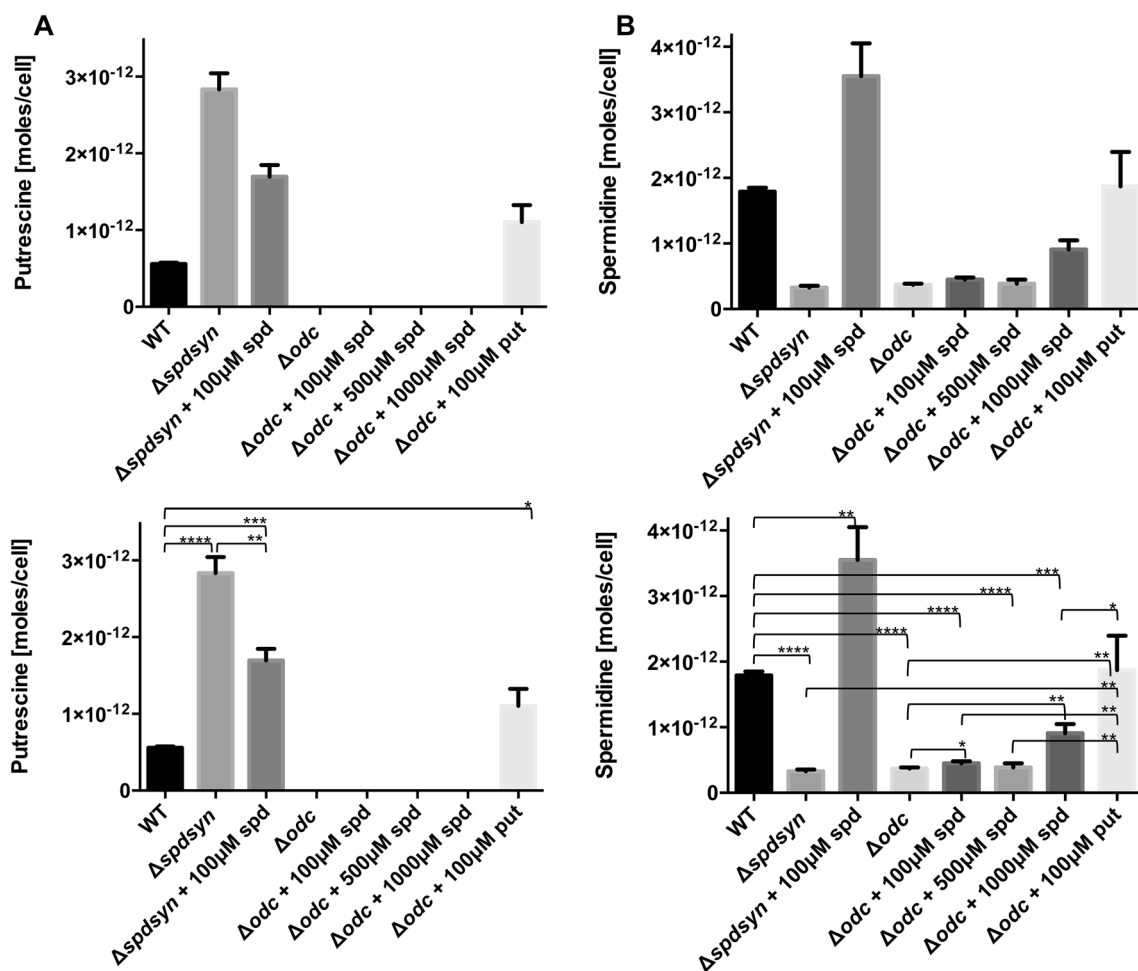
plemented with 100  $\mu M$  spermidine (gray triangles), 500  $\mu M$  spermidine (white diamonds), or 1000  $\mu M$  spermidine (black diamonds), or supplemented with 100  $\mu M$  putrescine (black triangle), and  $\Delta spdsyn$  parasites were incubated un-supplemented media (white squares) or supplemented with 100  $\mu M$  spermidine (black squares). Data are from two biological duplicates ( $n=2$ ) with error bars representing standard deviations. The experiment was repeated two more times with essentially the same outcome

then incubated in media without polyamines,  $\Delta odc$  parasites in the absence of polyamines, or in the presence of 100  $\mu M$  putrescine, or 100  $\mu M$ , 500  $\mu M$ , or 1000  $\mu M$  spermidine, and  $\Delta spdsyn$  parasites in the absence of polyamines or in the presence of 100  $\mu M$  spermidine. Parasites ( $1 \times 10^7$ ) were harvested after 3 days, washed three times in PBS, and extracted for polyamine pool determination with 10% trichloroacetic acid (TCA) as described previously (Jiang et al. 1999; Shim and Fairlamb 1988). An internal standard, 1,7-diaminoheptane, was added to the polyamine–TCA solutions for further processing. Samples were extracted in ethyl acetate and dried on a Speed Vac concentrator. The samples were derivatized with a solution of dansyl chloride (fluorescent label) and proline was added to scavenge the excess dansyl chloride. The derivatized polyamines were recovered with two ethyl acetate extractions, the organic layers were pooled and dried, and samples were dissolved in 200  $\mu l$  of 95% methanol/5% acetic acid. The polyamines were separated by high-performance liquid chromatography (flow rate = 1 ml/min) on a Shimadzu Prominence system equipped with a Phenomenex Kinetix C<sub>18</sub> XB reversed-phase column (100  $\times$  4.6 mm; 5  $\mu m$ ; 100  $\text{\AA}$ ). Gradient elution (45% B–80% B from 0 to 14 min; 80% B from 14 to 15 min) with solvent A (10 mM sodium phosphate; pH=7.2) and solvent B (acetonitrile) was used to separate putrescine (retention time  $\approx$  8.6 min), spermidine (retention time  $\approx$  13.9 min), and the internal standard 1,7-diaminoheptane (retention time  $\approx$  11.1 min). Fluorescence was measured on a Shimadzu RF-535 detector (340<sub>Ex</sub>/515<sub>EM</sub>). LabSolutions version 5.73 by Shimadzu

Corporation (Kyoto, Japan) software was used to calculate peak areas. A comparison of peak areas from samples to standard curves of putrescine and spermidine, which contained the internal standard 1,7-diaminoheptane, was used to quantify the individual polyamines. The experiments were conducted three times with biological triplicates for each experiment and the data (total  $n=9$  per cell line and condition) are shown in Fig. 4. The unpaired  $t$  test was used to evaluate statistical significance.

## Microscopy

Wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were allowed to reach stationary phase in media supplemented with no polyamines (wild-type), with 100  $\mu M$  putrescine ( $\Delta odc$ ), or with 100  $\mu M$  spermidine ( $\Delta spdsyn$ ). Parasites were then harvested, washed three times in PBS, and seeded in media with or without supplementation with polyamines. After 3 days, 500  $\mu l$  parasites were harvested, washed once in PBS, and 20  $\mu l$  samples were placed on poly-L-lysine-coated slides. Coverslips were applied without sealing. Bright-field images were taken on an AMG Evos XL at 40 $\times$  magnification within 10 min of adding samples to the slides. The experiment was repeated more than three times and visual observations were consistent. Cell body lengths, excluding the flagellum, were measured using an Olympus BX41 microscope at 40 $\times$  magnification utilizing the NeuroLucida software (MBF Biosciences). Three independent experiments were performed and 50–100 cells for each parasite



**Fig. 4** Polyamine levels in wild-type and mutant parasites in supplemented or un-supplemented media. Wild-type parasites were incubated in media without polyamines,  $\Delta odc$  parasites were incubated in the absence of polyamines, or in the presence of 100  $\mu$ M putrescine, 100  $\mu$ M, 500  $\mu$ M, or 1000  $\mu$ M spermidine, and  $\Delta spdsyn$  parasites were incubated in the absence of polyamines or in the presence of 100  $\mu$ M spermidine. Parasites were harvested after 3 days and polyamine levels were measured in wild-type and mutant parasites. **a** Shows putrescine levels and **b** depicts spermidine levels. The experi-

ments were conducted three individual times with three biological replicates for each experiment and the data (total  $n=9$  per cell line and condition) are shown with error bars representing standard deviations. The unpaired  $T$  test was performed using GraphPad software for statistical analysis, which is shown in the lower panels. A  $p$  value of  $<0.05$  is denoted with one asterisk, a  $p$  value of  $<0.01$  with two asterisks, a  $p$  value of  $<0.001$  with three asterisks, and a  $p$  value of  $<0.0001$  with four asterisks

strain and condition were measured in each experiment, with a total of 200–250 cells measured for each parasite strain and condition. The unpaired  $T$  test was performed using GraphPad software for statistical analysis.

### Viability assay

Wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were allowed to reach stationary phase in media supplemented with no polyamines (wild-type), with 100  $\mu$ M putrescine ( $\Delta odc$ ), or with 100  $\mu$ M spermidine ( $\Delta spdsyn$ ). Parasites were then harvested, washed three times in PBS, and seeded in media

with or without supplementation with polyamines. All cell lines were seeded at  $3 \times 10^5$ /ml and samples were taken daily. Viability was assessed on a Muse<sup>®</sup> Cell Analyzer (Millipore Sigma) using the Muse<sup>™</sup> Count and Viability reagent according to the manufacturer's manual. Briefly, Count and Viability reagent was added to each microcentrifuge containing parasite samples and incubated for 5 min at room temperature before inserting into the Muse<sup>®</sup> Cell Analyzer. Graphs were prepared using GraphPad. Data from three biological replicates ( $n=3$ ) are displayed in Fig. 6. The experiment was repeated two more times with essentially the same outcome.

## Rescue assay

Parasite survival was determined with a long-term rescue assay. Wild-type,  $\Delta odc$ , and  $\Delta spdsyn$  parasites were allowed to reach stationary phase in media supplemented with no polyamines (wild-type), with 100  $\mu\text{M}$  putrescine ( $\Delta odc$ ), or with 100  $\mu\text{M}$  spermidine ( $\Delta spdsyn$ ). Parasites were then harvested and washed three times in PBS. The  $\Delta odc$  mutants were seeded at  $5 \times 10^6$  cells/ml in 10 ml media without polyamine supplementation or in media containing 100  $\mu\text{M}$  spermidine. The  $\Delta spdsyn$  mutants were seeded at  $1 \times 10^6$  cells/ml in 10 ml media without polyamine supplementation. Multiple flasks were set up for each cell line and condition. Over the course of 18 weeks, weekly 500  $\mu\text{l}$  aliquots were taken from two flasks per cell line and condition, spun down, and pellets were resuspended in 1 ml media containing either 100  $\mu\text{M}$  putrescine ( $\Delta odc$ ) or 100  $\mu\text{M}$  spermidine ( $\Delta spdsyn$ ). The 1 ml parasite cultures were transferred into 24-well plates. One or two weeks after the rescue attempt, parasite survival was assessed under the microscope. Samples that showed healthy and motile parasites were deemed alive (rescued), while samples that showed non-motile forms were deemed dead. The experiment was repeated six times for  $\Delta odc$  and  $\Delta spdsyn$  mutants incubated in media without polyamine supplementation and three times for  $\Delta odc$  parasites incubated in media with 100  $\mu\text{M}$  spermidine. Each time, the rescue attempts were performed in duplicates.

## Results

### Polyamine requirements of gene deletion mutants

To establish  $\text{EC}_{50}$  values for putrescine and spermidine, and determine concentrations necessary for optimal growth of  $\Delta odc$  and  $\Delta spdsyn$  parasites, mutants were incubated in serial dilutions of 1000  $\mu\text{M}$  putrescine or 1000  $\mu\text{M}$  spermidine. Cell growth was assessed by measuring the conversion of resazurin to resorufin after 5 days (Fig. 2). The  $\Delta odc$  parasites required the addition of  $5.80 \pm 1.56$   $\mu\text{M}$  putrescine to the media for optimal growth, with an  $\text{EC}_{50}$  of  $1.88 \pm 0.54$   $\mu\text{M}$  (average of three experiments performed in duplicates). The downstream metabolite spermidine was not able to restore growth of  $\Delta odc$  parasites at concentrations up to 1000  $\mu\text{M}$ . The  $\Delta spdsyn$  parasites depended on  $55.00 \pm 7.07$   $\mu\text{M}$  spermidine supplementation for ideal growth, with an  $\text{EC}_{50}$  of  $11.5 \pm 9.19$   $\mu\text{M}$  (average of three experiments performed in duplicate). Supplementation of  $\Delta spdsyn$  parasites with putrescine, the substrate for the spermidine synthase reaction, did not rescue the growth defect of the  $\Delta spdsyn$  mutants. As  $\Delta spdsyn$  parasites are able to synthesize putrescine, the addition of putrescine was not expected to have an effect on growth.

### Proliferation of polyamine-starved parasites

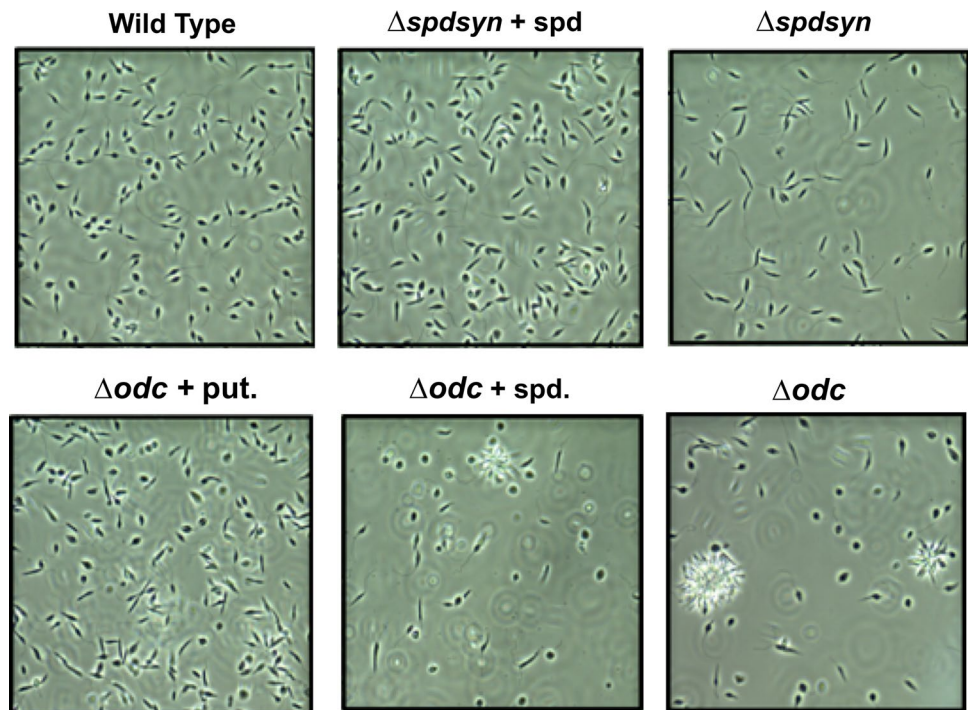
Proliferation of  $\Delta odc$  and  $\Delta spdsyn$  parasites incubated in polyamine-free or supplemented media was compared by counting cell numbers over the course of 7 days (Fig. 3). Wild-type parasites,  $\Delta odc$  mutants supplemented with 100  $\mu\text{M}$  putrescine, and  $\Delta spdsyn$  mutants supplemented with 100  $\mu\text{M}$  spermidine proliferated optimally over the course of the experiment (Fig. 3a). It should be noted that the supplemented  $\Delta spdsyn$  mutants showed slightly higher growth rates than the wild-type or supplemented  $\Delta odc$  mutants in Fig. 3a. However, growth curves of wild-type parasites and supplemented mutants were repeated numerous times and within the range of normal fluctuations these three cell lines proliferated equally well, which has also been reported previously (Boitz et al. 2009; Gilroy et al. 2011). In contrast, the  $\Delta odc$  mutants grown in polyamine-free media arrested cell division immediately (Fig. 3b). To investigate if this severe growth defect was due to the depletion of putrescine alone or both putrescine and spermidine, the  $\Delta odc$  mutants were incubated in the presence of 100  $\mu\text{M}$ , 500  $\mu\text{M}$ , or 1000  $\mu\text{M}$  spermidine. Some improvement in cell growth was observed in  $\Delta odc$  parasites in media supplemented with 500  $\mu\text{M}$  or 1000  $\mu\text{M}$  spermidine; however, it did not reach the level of growth observed in  $\Delta spdsyn$  mutants (Fig. 3b). In comparison with the  $\Delta odc$  mutants, the  $\Delta spdsyn$  parasites incubated in polyamine-free media exhibited an intermediate growth phenotype (Fig. 3b). The  $\Delta spdsyn$  parasites were initially seeded at  $5 \times 10^5$  parasites/ml and reached a density of about  $1 \times 10^7$  parasites/ml after 7 days, indicating that cells divided four to five times.

### Correlation of growth phenotypes with intracellular putrescine and spermidine levels

To correlate the observed growth deficits with intracellular putrescine and spermidine levels, polyamine pools were measured in wild-type and mutant parasites. Wild-type cells were incubated in media without supplementation, and the  $\Delta odc$  and  $\Delta spdsyn$  gene deletion mutants were incubated in media with or without polyamine supplementations for 3 days before parasites were harvested for the analysis of intracellular polyamine content.

Intracellular putrescine levels were below the detection level in  $\Delta odc$  parasites incubated in polyamine-free media or in media containing various concentrations of spermidine (Fig. 4). When the  $\Delta odc$  mutants were incubated in media supplemented with 100  $\mu\text{M}$  putrescine, the intracellular putrescine levels were about twofold higher than in wild-type parasites, suggesting that a robust uptake of putrescine occurred in these cells to counter the inability to synthesize putrescine. Putrescine levels were about three- and fivefold higher in  $\Delta spdsyn$  mutants incubated in media

**Fig. 5** Morphology of wild-type and mutant parasites in supplemented or un-supplemented media. Bright-field images of parasites were taken on an Evos microscope after 3 days of incubation. Wild-type parasites were grown in media without polyamine supplementation,  $\Delta spdsyn$  were grown without polyamines or in the presence of 100  $\mu\text{M}$  spermidine, and  $\Delta odc$  with incubated in the presence of 100  $\mu\text{M}$  putrescine, 100  $\mu\text{M}$  spermidine or no polyamine supplementation. The experiment was repeated more than three times and visual observations were consistent



with or without spermidine, respectively, than in wild-type parasites. This observation can likely be ascribed to an accumulation of putrescine due to the inability of  $\Delta spdsyn$  mutants to convert putrescine to spermidine.

Intracellular spermidine levels in the  $\Delta odc$  and  $\Delta spdsyn$  parasites incubated in spermidine-free media were considerably lower than in wild-type parasites. However, in contrast to the profound depletion of putrescine levels in  $\Delta odc$  mutants (below detection level), spermidine levels in both  $\Delta odc$  and  $\Delta spdsyn$  parasites were only about five-fold lower than in wild-type parasites. Supplementation of  $\Delta spdsyn$  mutants with 100  $\mu\text{M}$  spermidine resulted in a twofold increase of spermidine levels compared to wild-type parasites, suggesting robust uptake to counter the inability to synthesize spermidine. In contrast, supplementation of  $\Delta odc$  parasites with 100, 500, or 1000  $\mu\text{M}$  spermidine did not restore spermidine levels to that of wild-type parasites. In fact, only supplementation of  $\Delta odc$  parasites with 1000  $\mu\text{M}$  spermidine caused a modest increase of spermidine levels compared to those of  $\Delta odc$  parasites incubated in polyamine-free media.

Taken together, the analysis of intracellular polyamine levels revealed that both mutant cell lines,  $\Delta odc$  and  $\Delta spdsyn$ , contained low levels of intracellular spermidine but only the  $\Delta spdsyn$  mutants contained putrescine. Thus, the immediate cessation of cellular proliferation and other phenotypic observations in the starved  $\Delta odc$  cell line (described below) correlated with the loss of putrescine.

### Morphology of polyamine-starved parasites

The effect of polyamine deficiencies on parasite morphology was assessed. The  $\Delta odc$  mutants incubated in polyamine-free or spermidine-supplemented media exhibited a more rounded morphology (Fig. 5), were less motile (data not shown) and more prone to form clusters compared to wild-type or putrescine-supplemented  $\Delta odc$  parasites. In contrast,  $\Delta spdsyn$  parasites incubated in media without spermidine showed cell shapes and motility similar to wild-type or supplemented  $\Delta spdsyn$  mutants (Fig. 5). Cell body lengths (without flagella) were measured and compared for the different cell lines and supplement conditions after 3 days of starvation. The body length of putrescine-depleted  $\Delta odc$  parasites averaged  $6.27 \pm 1.17 \mu\text{m}$ , while putrescine-supplemented  $\Delta odc$  and wild-type parasites exhibited lengths of  $11.88 \pm 1.43 \mu\text{m}$  and  $14.41 \pm 0.17 \mu\text{m}$ , respectively (Table 1). In contrast, the body length of spermidine-depleted  $\Delta spdsyn$  parasites,  $12.51 \pm 1.18 \mu\text{m}$ , was similar to that of spermidine-supplemented  $\Delta spdsyn$ ,  $12.22 \pm 0.63 \mu\text{m}$ , and wild-type parasites,  $14.41 \pm 0.17 \mu\text{m}$ . Statistical analysis (unpaired student *t* test) confirmed that the differences between the body lengths of the putrescine-depleted  $\Delta odc$  parasites and the putrescine-supplemented  $\Delta odc$  parasites or wild-type parasites were statistically significant.

### Viability of polyamine-starved parasites

The putrescine-starved  $\Delta odc$  parasites ceased proliferation immediately (Fig. 3), demonstrated a rounded morphology



**Table 1** Body length of wild-type and mutant parasites

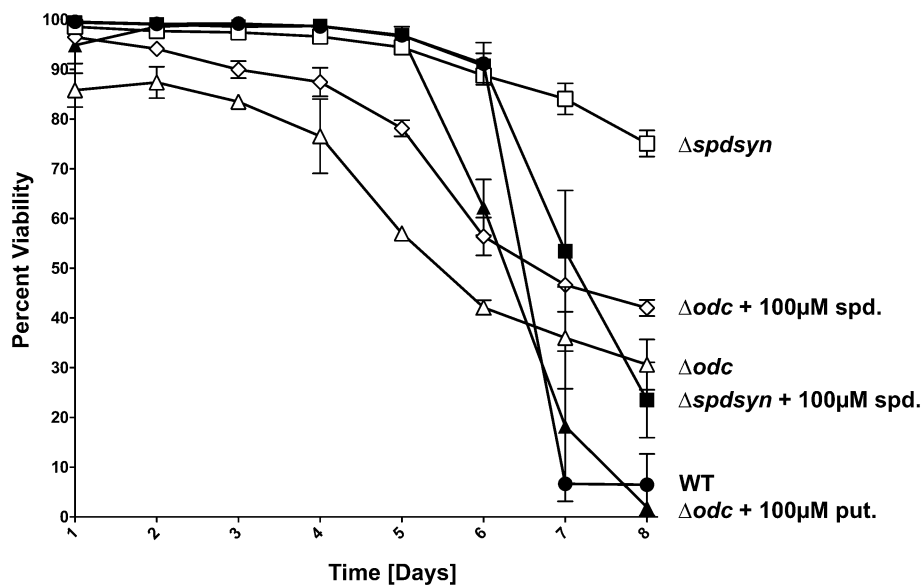
| Cell line and supplement                             | Cell length in $\mu\text{m}$<br>Mean (average) $\pm$ standard deviation | Total number of cells measured |
|--|---|--------------------------------|
| WT   | 14.41 $\pm$ 0.17  | 200                            |
| $\Delta\text{odc}$ + 100 $\mu\text{M}$ putrescine    | 11.88 $\pm$ 1.43  | 200                            |
| $\Delta\text{odc}$                                   | 6.27 $\pm$ 1.17   | 250                            |
| $\Delta\text{odc}$ + 100 $\mu\text{M}$ spermidine    | 7.24 $\pm$ 0.72   | 200                            |
| $\Delta\text{spdsyn}$ + 100 $\mu\text{M}$ spermidine | 12.22 $\pm$ 0.63  | 200                            |
| $\Delta\text{spdsyn}$                                | 12.51 $\pm$ 1.18  | 250                            |

Wild-type parasites were grown in media without polyamine supplementation,  $\Delta\text{spdsyn}$  parasites were grown without polyamines or in the presence of 100  $\mu\text{M}$  spermidine, and  $\Delta\text{odc}$  mutants were incubated in the presence of 100  $\mu\text{M}$  putrescine, 100  $\mu\text{M}$  spermidine or no polyamine supplementation. Parasite body length was measured after 3 days of starvation as described in “Materials and methods”. The table represents data from three independent experiments, and 50–100 cells for each parasite strain and condition were measured in each experiment. Statistical analysis using the unpaired *T* test revealed significant differences (*p* value < 0.001) for  $\Delta\text{odc}$  mutants incubated with or without spermidine when compared to the size of wild-type parasites or  $\Delta\text{odc}$  mutants incubated in the presence of putrescine

Wild-type parasites,  $\Delta\text{spdsyn}$  parasites supplemented with spermidine, and  $\Delta\text{odc}$  parasites supplemented with putrescine showed a high percentage of viable parasites, ~90%, up to day 4 or 5 (Fig. 6). Once the culture reached stationary phase and a high density, viability plummeted rapidly (Fig. 6). The  $\Delta\text{spdsyn}$  mutants grown in the absence of polyamines sustained a high viability of above 70% even after 8 days of starvation, while the  $\Delta\text{odc}$  parasites with or without supplementation of spermidine showed a gradual decline in viability, starting by day 2 or 3, and reached a low 45% ( $\Delta\text{odc}$  with spermidine) and 30% ( $\Delta\text{odc}$ ) viability by day 8 (Fig. 6). The addition of 100  $\mu\text{M}$  spermidine improved viability of the  $\Delta\text{odc}$  parasites slightly. This analysis demonstrates that the putrescine-depleted  $\Delta\text{odc}$  parasites not only ceased proliferation, they also showed a steady decrease of cell viability under polyamine starvation conditions.

### Assessment of long-term survival of gene deletion mutants

Parasite survival was determined over the course of several weeks by a rescue assay. For this assay, parasites were incubated in starvation conditions and samples were taken once



**Fig. 6** Viability of wild-type and mutant parasites in supplemented or un-supplemented media. Parasites were seeded at  $3 \times 10^5$  parasites/ml and samples were analyzed by flow cytometry for viability every day for the course of 8 days. Wild-type parasites were incubated in un-supplemented media (black circles),  $\Delta\text{odc}$  parasites were incubated in un-supplemented media (white triangles), or supplemented

with 100  $\mu\text{M}$  spermidine (white diamonds), or 100  $\mu\text{M}$  putrescine (black triangles), and  $\Delta\text{spdsyn}$  parasites were incubated in un-supplemented media (white squares) or supplemented with 100  $\mu\text{M}$  spermidine (black squares). Data are from three biological replicates (*n* = 3) with error bars representing standard deviations. The experiment was repeated two more times with essentially the same outcome

(Fig. 5 and Table 1), and most parasites appeared to be non-motile (data not shown). However, as these observations did not discern whether parasites were alive or dead, the percentage of viable parasites was determined by flow cytometry.

a week. Rescue was attempted by the addition of putrescine or spermidine to the  $\Delta\text{odc}$  and  $\Delta\text{spdsyn}$  parasite cultures, respectively. The  $\Delta\text{odc}$  parasites incubated in polyamine-free

**Table 2** Long-term survival of polyamine-starved parasites

| Cell line and supplement              | Survival in weeks<br>Mean (average) $\pm$ standard deviation | Number of experiments |
|---------------------------------------|--|-----------------------|
| $\Delta odc$                          | 2.08 $\pm$ 0.51  | 6                     |
| $\Delta spdsyn$                       | 5.92 $\pm$ 0.67  | 6                     |
| $\Delta odc$ + 100 $\mu$ M spermidine | 14.17 $\pm$ 2.14   | 3                     |

The  $\Delta spdsyn$  and  $\Delta odc$  mutants were incubated in media without polyamine supplementation and  $\Delta odc$  mutants were also incubated in media with 100  $\mu$ M spermidine. Once a week, aliquots were taken and rescued by the addition of putrescine ( $\Delta odc$ ) or spermidine ( $\Delta spdsyn$ ) as outlined in “Materials and methods”. The experiment was repeated six times for  $\Delta odc$  and  $\Delta spdsyn$  mutants incubated in media without polyamine supplementation and three times for  $\Delta odc$  parasites incubated in media with 100  $\mu$ M spermidine. Each time the rescue attempts were performed in duplicates

media survived for only approximately 2 weeks; and could not be rescued by the addition of 100  $\mu$ M putrescine after that time (Table 2). In contrast, the addition of 100  $\mu$ M spermidine allowed  $\Delta odc$  mutants to persist for 14 weeks (Table 2). The  $\Delta spdsyn$  parasites incubated in polyamine-free media showed an intermediate survival phenotype and lived for 6 weeks in a quiescent-like state (Table 2).

## Discussion

Our studies demonstrate that both polyamines, putrescine and spermidine, are essential for *Leishmania* and that putrescine has previously unrecognized vital functions beyond its role as a precursor for spermidine formation. Putrescine-depleted  $\Delta odc$  parasites showed a more profound decrease in proliferation and viability compared to putrescine-rich  $\Delta spdsyn$  parasites, pointing towards specific functions of putrescine for these cellular processes.

Spermidine is the final product of the polyamine biosynthetic pathway in *Leishmania* (Fig. 1) and has been proposed to be the only essential polyamine (Heby et al. 2007; Jiang et al. 1999; Reguera et al. 2009). Indeed, studies have demonstrated that the sole essential function of the amino acid ornithine is as precursor for the polyamine biosynthesis in *Leishmania* (Boitz et al. 2016; da Silva and Floeter-Winter 2014; da Silva et al. 2012; Reguera et al. 2009; Roberts et al. 2004). Similarly, it has been suggested that putrescine is merely the precursor metabolite for spermidine formation, and supplementation of spermidine was found to be sufficient to at least partially restore growth in *L. major*  $\Delta arg$  and *L. donovani*  $\Delta odc$  mutants (Jiang et al. 1999; Reguera et al. 2009). However, a recent study found that *L. donovani*  $\Delta arg$  mutants did not proliferate in the presence of spermidine (Boitz et al. 2016) and we now report that supplementation

of up to 1000  $\mu$ M spermidine did not rescue *L. donovani*  $\Delta odc$  mutants. Previous observations that spermidine was able to partially restore growth of *L. major*  $\Delta arg$  or *L. donovani*  $\Delta odc$  mutants may have been caused by putrescine contaminations of commercially available spermidine. This conjecture is plausible as the  $EC_{50}$  values for putrescine were 7.5  $\mu$ M for *L. donovani*  $\Delta arg$  (Boitz et al. 2016) and 1.88  $\mu$ M for *L. donovani*  $\Delta odc$  mutants (Fig. 2a), and thus very small amounts of putrescine contamination could be sufficient to allow at least some growth. Our observation that *L. donovani*  $\Delta odc$  mutants showed a slight increase in growth at 500 and 1000  $\mu$ M (Fig. 2a) can possibly also be ascribed to small amounts of putrescine in the spermidine stock solution. The more recent observations that *L. donovani*  $\Delta arg$  (Boitz et al. 2016) and  $\Delta odc$  mutants (Fig. 2) require putrescine supplementation is profound as it shifts the current paradigm of the importance of putrescine from being a precursor metabolite to having essential functions on its own. Intriguingly, similar observations have been made in *T. brucei*, where RNAi-mediated silencing of ODC could be rescued by supplementation with putrescine but not spermidine and silencing of ODC resulted in a more rapid cell death than silencing of SPDSYN (Xiao et al. 2009). Although this observation was ascribed to the lack of both putrescine and spermidine, it is feasible that putrescine has a uniquely important function for trypanosomatids.

The experiments presented here reveal that putrescine is essential for parasite proliferation as putrescine-depleted  $\Delta odc$  mutants displayed an immediate cessation of proliferation, while putrescine-rich  $\Delta spdsyn$  mutants exhibited an intermediate proliferation phenotype compared to the  $\Delta odc$  mutants (Fig. 3). As both cell lines contained similar, albeit low, amounts of spermidine (Fig. 4), the immediate cessation of proliferation in the  $\Delta odc$  mutants can be attributed to the lack of putrescine. In mammalian cells, polyamines have been linked to cellular proliferation (Bachrach et al. 2001; Igarashi and Kashiwagi 2000, 2010; Miller-Fleming et al. 2015; Minois et al. 2011; Pegg 2016); however, the contributions of putrescine, spermidine, or spermine are difficult to discern as mammalian cells have catabolic pathways that can convert spermine and spermidine to putrescine (Bachrach et al. 2001; Igarashi and Kashiwagi 2010; Seiler 2004; Seiler et al. 1981). Because *Leishmania* parasites lack a back-conversion pathway to recover lost pools of putrescine, our results show for the first time an unambiguous function of putrescine for cellular proliferation.

As stated above, mutant parasites incubated in polyamine-free media contained spermidine (Fig. 4). Previous measurements of intracellular pools in *L. donovani*  $\Delta odc$  and  $\Delta spdsyn$  knockout cell lines also showed that parasites maintained low but stable levels of spermidine over several days of starvation (Jiang et al. 1999; Roberts et al. 2001). Similarly, studies in *L. major*  $\Delta arg$  mutants and arginine-depleted

*L. donovani* wild-type parasites found a marked decrease of putrescine levels while spermidine pools diminished at a slower rate (Mandal et al. 2016; Reguera et al. 2009). Spermidine is an essential metabolite for the hypusination and activation of eIF5A (Chawla et al. 2010, 2012) and the formation of trypanothione (Colotti and Ilari 2011; Ilari et al. 2017). It is conceivable that less spermidine is being used for these downstream reactions to maintain spermidine pools. It is also possible that spermidine is formed from trypanothione as TRYS is a bifunctional enzyme catalyzing the biosynthesis and hydrolysis of trypanothione (Fyfe et al. 2008). Although, we have previously shown that trypanothione levels in starved *L. donovani*  $\Delta odc$  and  $\Delta spdsyn$  parasites indeed plummet over time (Jiang et al. 1999; Roberts et al. 2001), it is unknown whether this is due to a lack of trypanothione formation or conversion of trypanothione to spermidine. Regardless, the observation that polyamine-starved parasites maintain low but stable levels of spermidine underscore the importance of this polyamine.

Interestingly, a comparison of  $\Delta odc$  and  $\Delta spdsyn$  parasites incubated in spermidine-supplemented media showed a profound difference in spermidine levels (Fig. 4), although both cell strains would have been expected to compensate for the loss of spermidine biosynthesis by increased uptake. While the  $\Delta spdsyn$  mutants incubated in 100  $\mu\text{M}$  spermidine exhibited a twofold increase in spermidine levels compared to spermidine levels in wild-type cells,  $\Delta odc$  parasites incubated with 100  $\mu\text{M}$ , 500  $\mu\text{M}$ , or 1000  $\mu\text{M}$  spermidine displayed spermidine levels that were lower than in wild-type parasites (Fig. 4). Thus,  $\Delta spdsyn$  parasites markedly increased spermidine uptake while  $\Delta odc$  parasites did not, which leads to intriguing speculations about the molecular mechanisms regulating spermidine transport. Perhaps, increased transport of spermidine in  $\Delta spdsyn$  parasites is regulated by loss of SPDSYN activity or metabolite flux rather than levels of metabolite pools. This conjecture is supported by a previous study on arginine transport in *L. donovani*  $\Delta odc$  and  $\Delta spdsyn$  parasites, which showed a significant reduction in arginine transport, even in the presence of putrescine or spermidine (Darlyuk et al. 2009). An alternative explanation for our observation that  $\Delta odc$  parasites exhibited only little increase in uptake of spermidine may be that the  $\Delta odc$  parasites, after 3 days of starvation, were already compromised in health and were thus not capable of robust uptake of spermidine.

Differences in morphology and motility between the two mutant strains were also substantial. While the starved  $\Delta odc$  parasites rounded up and clumped together within a few days, the starved  $\Delta spdsyn$  mutants exhibited morphology similar to that of wild-type parasites and supplemented mutants throughout several days of starvation (Fig. 5). The observation that putrescine depletion caused this severe

stress phenotype further supports our hypothesis that putrescine fulfills important roles in parasites.

Both  $\Delta odc$  and  $\Delta spdsyn$  mutants perished in media without the supplementation of putrescine or spermidine, respectively, although at different times. The  $\Delta odc$  parasites showed reduced viability within a few days (Fig. 6) and died after 2 weeks (Table 2). It is likely that the small amount of intracellular spermidine present after 3 days of starvation was depleted after 2 weeks, and that the lack of both polyamines caused cell death. In contrast,  $\Delta spdsyn$  parasites maintained a high level of viability (over 70%), during 8 days of starvation (Fig. 6), and were able to survive for an average of 6 weeks before cell death occurred (Table 2). The presence of putrescine alone appeared to be sufficient to allow  $\Delta spdsyn$  parasites to enter a quiescent-like state. Surprisingly, the addition of 100  $\mu\text{M}$  spermidine to  $\Delta odc$  parasites enabled the mutants to survive for 14 weeks (Table 2). Thus, although the supplementation of  $\Delta odc$  parasites with spermidine had only a marginal effect on proliferation (Fig. 3) or initial viability (Fig. 6), it had a profound effect on parasite survival. Further experiments are necessary to quantitate differences in cell death in these cultures, and to determine what types of adaptations or mutations allowed persistence. Spermidine has been implied to have a role in longevity in mammalian cells, yeast, and nematodes (Madeo et al. 2010; Morselli et al. 2009; Petrovski and Das 2010), and polyamines may have a similar effect in *Leishmania* parasites. Thus, while both polyamines were essential for ultimate parasite survival, the presence of either putrescine or spermidine alone may allow parasites to survive for several weeks in a quiescent-like state. The intracellular mechanism and reprogramming associated with this phenomenon could be of clinical importance, as *Leishmania* parasites may also persist in infected patients (Bogdan 2008; Mandell and Beverley 2017). While some studies have investigated how the human immune system suppresses parasite numbers, very few investigations have explored how the parasite is able to persist.

It is of interest to note that the proliferation and survival discrepancies between the  $\Delta odc$  and  $\Delta spdsyn$  mutants mirrored in vivo infectivity phenotypes. While  $\Delta odc$  parasites exhibit profoundly diminished infectivity in mice compared to wild-type parasites (Boitz et al. 2009), the  $\Delta spdsyn$  parasites show a less pronounced, although significant, reduction in intracellular survival (Gilroy et al. 2011). We previously postulated that putrescine salvage is severely limited in the phagolysosome (Boitz et al. 2016). Together, these observations suggest that putrescine is a key metabolite for both promastigotes and intracellular amastigotes, and validate the polyamine biosynthetic enzyme ODC as a promising therapeutic target.

## Conclusions

Collectively, our observations confirm a previous report that putrescine is not merely a precursor metabolite for spermidine formation and, furthermore, suggest that putrescine has specific functions for parasite proliferation and viability. While the polyamine biosynthetic pathway has already been endorsed as a potential therapeutic target, our studies highlight ODC inhibition and putrescine depletion as the most promising strategy. In addition, our results suggest that both polyamines are essential for parasite survival but that the presence of either putrescine or spermidine alone may have allowed parasites to survive in a quiescent-like state for several weeks. The  $\Delta odc$  and  $\Delta spdsyn$  mutants are valuable model systems to not only study the functions of putrescine but also to elucidate processes vital for proliferation and for intracellular reprogramming associated with survival versus cell death, which may ultimately lead to new therapeutic strategies.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human participants and/or animals** This research did not involve human participants or animals.

**Informed consent** None.

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