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Division of the single mitochondrion in *Trypanosoma brucei* and its impact on the cell cycle

THESE

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SUMMARY

Trypanosoma brucei is one of the earliest diverging eukaryotes with a bona fide mitochondrion. In contrast to most other eukaryotes, it has a single mitochondrion only which shows a large network-like morphology. This unique feature makes *T. brucei* an excellent model to study some unique aspects of mitochondrial biology.

In the first part of this thesis, we used *T. brucei* as a model to study the mechanisms of mammalian apoptosis. Additionally to the one unit characteristic of its mitochondrion, the trypanosomes lack all components of the "classical" apoptotic machinery. Because of these two unique features, we were able to temporally separate the three major mitochondrial events that are induced by Bax expression during apoptosis. First, cytochrome *c* is released from the mitochondrial intermembrane space. This event is followed by a loss of the membrane potential and finally by mitochondrial fragmentation. Interestingly, all these events are reversible when Bax is removed.

The next two sections of the thesis focus on the mechanism of division of the mitochondrion in *T. brucei*. The proteins involved in this process are well conserved and two of them are found in the genome of *T. brucei*. We show that the single dynamin-like protein (TbDLP), normally specialized in mitochondrial fission, is not only involved in mitochondrial division, but also required for endocytosis, a process normally mediated by classical dynamins which are absent in *T. brucei*. The two specific intracellular localizations of TbDLP confirm the dual function of this protein. Moreover, we showed that mitochondrial fission is required for the completion of cytokinesis in *T. brucei*, suggesting that mitochondrial fission might be a checkpoint for cell division. Finally, we have also identified the putative Fis1 homologue of *T. brucei*.

Generally in eukaryotes, the mitochondrial DNA is distributed all over the matrix. However in *T. brucei*, it is restricted to a discrete structure termed the kinetoplast (or kDNA). In the last part of this thesis we show that TbMiX, a protein of the outer mitochondrial membrane, is essential for the correct positioning of the kDNA. Moreover, we present evidence that this protein may link the mitochondrion to the subpellicular cytoskeleton of *T. brucei*, suggesting that the microtubules present in this structure are required to determine the position of the kDNA.

RESUME

Trypanosoma brucei est l'un des plus anciens organismes à posséder de véritables mitochondries. Contrairement aux autres eucaryotes, il ne contient qu'une seule de ces organelles. Cette caractéristique unique fait de *T. brucei* un excellent système pour étudier certains aspects spécifiques de la biologie mitochondriale.

Dans la première partie de cette thèse, nous avons utilisé *T. brucei* comme modèle afin d'étudier les mécanismes de l'apoptose des cellules mammifères. En plus de la présence d'une mitochondrie unique, aucun composant du processus conventionnel de l'apoptose n'est présent chez les trypanosomes. Grâce à ces deux caractéristiques, nous avons pu séparer dans le temps les trois événements majeurs liés à la mitochondrie qui sont induits par l'expression de la protéine pro-apoptotique Bax pendant l'apoptose. Tout d'abord, le cytochrome *c* est libéré de l'espace intermembranaire mitochondrial. Suivent ensuite la perte du potentiel de membrane et finalement la fragmentation de la mitochondrie. Il est intéressant de voir que tous ces événements sont réversibles si Bax est retiré du milieu.

Les deux sections suivantes de la thèse se focalisent sur le mécanisme régulant la division des mitochondries chez *T. brucei.* Les protéines impliquées dans ce processus ont été bien conservées durant l'évolution et on trouve deux d'entre elles dans le génome de *T. brucei*. Les protéines similaires aux dynamines (DLP, pour dynamin-like protein) sont en général impliquées dans le mécanisme de division des mitochondries. Nous avons découvert que chez *T. brucei*, l'unique DLP présente est aussi requise pour l'endocytose, un processus qui est normalement effectué par les dynamines « classiques », absentes dans cet organisme. La localisation intracellulaire a révélé la présence de DLP à deux endroits spécifiques, ce qui confirme cette double fonction. De plus, nous avons démontré que la division des mitochondries est essentielle pour l'accomplissement de la cytokinèse chez *T. brucei*. Ce dernier point suggère que la division des mitochondries pourrait être un point de contrôle pour la progression du cycle cellulaire. Finalement, nous avons également identifié un homologue potentiel de Fis1 chez *T. brucei*.

Généralement chez les eucaryotes, l'ADN mitochondrial est distribué dans toute la matrice de l'organelle. Cependant chez *T. brucei*, celui-ci est limité à une structure discrète appelée le kinétoplaste. Dans la dernière partie de cette thèse, nous montrons que TbMiX, une protéine de la membrane externe de la mitochondrie, est essentielle pour le positionnement correct du kinétoplaste. De plus, nous présentons des indices selon lesquels cette protéine pourrait lier la mitochondrie au cytosquelette de *T. brucei*, ce qui suggère que les microtubules présents dans cette structure sont requis pour la détermination de la position de l'ADN mitochondrial.

I. INTRODUCTION

1. Apoptosis

Apoptosis is an essential physiological process of programmed cell death (PCD) in multicellular organisms. This well-organized mechanism promotes cellular suicide of specific cells to confer advantages to the whole organism. For example, apoptosis has been shown to be required for the separation of the fingers during human embryo development. The process is also required for tissue homeostasis, meaning that the turnover of cells in different tissues such as blood or skin must be counterbalanced with cell death to maintain a more or less constant number of cells. This turnover amounts to about 50 to 70 billion cells per day in an adult human. Moreover, the ability of cells to commit suicide is a very important mechanism for the proper functioning of the immune system. It allows the elimination of defective cells that have accumulated mutations and of cells that are infected by viruses. Thus, misregulation of apoptosis can contribute to various human diseases, such as cancers, autoimmune diseases and neurodegenerative disorders (1).

PCD is not restricted to apoptosis, but also includes autophagy. In this process, contrary to apoptosis where the organelles for the most part retain their integrity, the cell compartmentalizes and forms large vacuoles that consume organelles in a predefined order, the nucleus being the last (2). A third mechanism of cell death, generally considered as unprogrammed, is termed necrosis. This process is often a consequence of cell injury. The main difference with between necrosis on one side and apoptosis and autophagy on the other side is that during necrosis, the organelles dissolve and the plasma membrane ruptures. This releases intracellular components into the surrounding tissues, which leads to an immune response. In PCD in contrast, the dying cell forms vesicles to avoid the release of cytoplasmic material (in the case of apoptosis) or cell lysis is prevented (in the case of autophagy). Furthermore the cell displays phosphatidylserine on its outer surface to promote its phagocytosis (3).

In the following chapter, I will focus on the mechanism of apoptosis in mammalian cells, but at the same time I will also consider the process in unicellular organisms, where its function is not obvious. Finally, I will outline the advantages of studying apoptosis in such unicellular organisms.

a) Apoptosis in mammalian cells

There are two main forms of how apoptosis can be induced in mammalian cells: the intrinsic and the extrinsic pathways. The intrinsic pathway activates PCD in response to stress signals from the inside of the cell, such as DNA damage or nutrient deprivation. The extrinsic pathway on the other hand is activated through the binding of extracellular ligands to "death receptors" at the plasma membrane. This binding leads to the assembly of a death-inducing signaling complex (DISC) that is able to activate death proteases, termed caspases (cystein protease with aspartate substrate specificity). At the end, both pathways converge in caspase cascades that ultimately result in DNA degradation, the final step of apoptosis (Fig.1) (1).

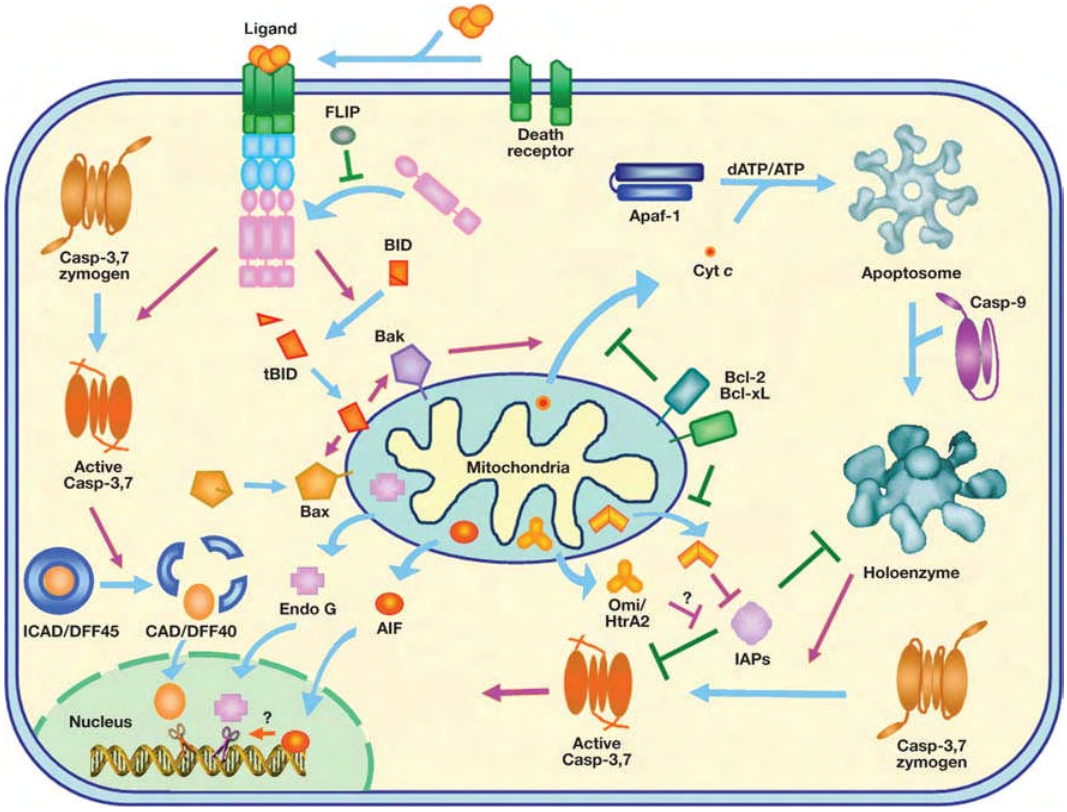


Figure 1: Schematic representation of the apoptotic pathways in mammalian cells. The cyan arrows indicate signal flow. Pro- and anti-apoptotic activities are colored magenta and green respectively. See text for detail (1).

Mitochondria are major actors in the intrinsic pathway of apoptosis (3-6). Indeed this organelle has been shown to release important factors for the activation of caspases during PCD. This release is mediated by members of the Bcl-2 proteins family, including Bax, Bcl-x_L and Bid. In non-apoptotic cells, the pro-apoptotic Bax remains mainly in the cytosol in an inactivated form, probably due to its binding to Bcl-x_L (5, 6). But when apoptosis is initiated, Bcl-x_L is dissociated from Bax, which can then form homo-oligomeres. This allows its translocation to the outer mitochondrial membrane (OMM). The mechanisms initiating the activation of Bax are not clearly understood yet and several different ones have been proposed. For example, the transcription factor p53 has been shown to not only promote the expression of genes involved in apoptosis in response to DNA damage, but also to directly activate Bax, through its binding to Bcl-x_L (5) . Bid, another member of the Bcl-2 family, on the other hand is known to promote the activation of Bax through direct binding (5, 7). Interestingly, Bid can also be activated through the extrinsic pathway and thus links the two pathways of apoptosis induction (1).

Once Bax is present on the mitochondria, it causes the release of cytochrome *c* from the intermembrane space. However the mechanism of this Bax-induced permeabilization is still unknown. Three hypotheses have been proposed. The first one suggests that Bax translocation could initiate swelling of the mitochondrial matrix and distortion of the inner mitochondrial membrane (IMM). This process activates the permeability transition pore (PTP) and eventually leads to the rupture of the OMM and thus to cytochrome *c* release. The second theory is based on the ability of Bax and Bcl-x_L to form pores in liposomes and thus suggests that Bax, alone or in association with VDAC or the PTP, promotes pores formation in the OMM to release small proteins such as cytochrome *c* (3-5). Finally, the third hypothesis proposes that Bax recruits the mitochondrial fission apparatus to permeabilize the OMM. Indeed it has been shown that Drp1, a major component of this machinery, is essential for apoptosis and cytochrome *c* release (8). Furthermore, Bax is colocalized with Drp1 on the OMM during apoptosis (9).

Finally, once in the cytosol, the cytochrome *c* can bind Apaf-1 (apoptotic protease-activating factor 1) to form, in presence of dATP or ATP, a multimeric complex termed the “apoptosome”. This complex is then able to activate the caspase cascade, that ultimately leads to DNA degradation (1, 3).

Mitochondria in apoptotic cells not only release cytochrome *c* but also other factors such as Smac/Diablo or HtrA2 that can neutralize inhibitors of proapoptotic proteins. Other proteins of the intermembrane space are AIF (apoptosis-inducible factor) and Endonuclease G. When they are released, they translocate to the nucleus to promote direct DNA fragmentation in a caspase-independent pathway (4). Finally, as a confirmation of the importance of mitochondria in apoptosis, it has been shown that these organelles invariably change their morphology during PCD from a reticular network to vesicular punctiform structures, in process that is Drp1-dependent. Interestingly, caspase inhibitors do not affect this mitochondrial fragmentation, whereas apoptosis on the other hand requires this change of mitochondrial morphology (10).

b) Apoptosis in unicellular organisms

Whereas apoptosis makes a lot of sense in multicellular organisms, the advantages of this process for unicellular organisms are much less evident. However, apoptosis or apoptosis-like phenotypes have been found in many different single-cell eukaryotes such as yeast (11-16), *Kinetoplastidae* (17-21), *Tetrahymena* (22-24) and other organisms such as *Dictyostelium* (25, 26). It has been also suggested that PCD also occurs in bacteria (27-29). So what is the benefit for a unicellular organism to commit suicide? It has been shown that populations of unicellular organisms are often clonal and can be found in complex communities that in many ways look like multicellular organisms. Bacteria for example have been shown to secrete pheromones that induced simultaneous change gene expression. Furthermore, *Dictyostelium* is known for its ability to regroup in a multicellular-like organism. Accordingly, considering the community aspect, committing suicide could potentially limit the spread of viral infections or pathogens, or reduce the amount of cells with damaged DNA to maintain a low mutation rate in the population. Moreover, apoptosis is a potential response to nutrient deprivation and to bacterial overpopulation of the medium. Cell suicide can also be required for the normal course of development (13, 25, 30, 31). So death of some unicellular organisms is a dramatic cost for the organism itself, but can lead to great benefit at the community level.

Yeast

Yeast, whose genome does not encode any orthologues of the classical mammalian apoptotic machinery, can undergo programmed cell death, showing the typical apoptotic changes. Indeed, it has been showed that in presence of reactive oxygen species such as H_2O_2, yeast shows DNA fragmentation, phosphatidyl serine externalization and chromatin condensation (11-13). All these events are markers of mammalian apoptosis. Some yeast mutants show the apoptotic phenotype even in absence of induction. In the first one that has been characterized it was the CDC48 protein, an AAA ATPase involved in vesicular fusion , that was affected (11, 12). One of the most interesting finding was the identification of a yeast metacaspase (12). This caspase-related protease clearly related to mammalian caspases, termed YCA1, is cleaved in a caspase-typical way and displays a caspase-like proteolytic activity. Moreover, disruption of YCA1 prevents the apoptotic response to H_2O_2, whereas over-expression of the protein strongly stimulates the caspase-like activity (13). In summary these observations suggest that YCA1 functions as a bona fide caspase. More recently other orthologues of the mammalian apoptotic pathway, such as AIF (14) or HtrA2 (15), have been discovered in yeast. Several mutants have also been shown to not suppress, but only delay apoptosis (11, 12). Finally, it was shown that, as in mammalian cells, the conserved proteins that are required for mitochondrial fission (see point 2 of the introduction), are also involved in the yeast apoptotic pathway (16).

Kinetoplastidae

No homologues of proteins involved in apoptosis have been found in *Kinetoplastidae*. However apoptosis-like processes resulting in DNA fragmentation have been described in *Leishmania major* (17), *Leishmania donovani* (18), *Trypanosoma cruzi* (19) and *Trypanosoma brucei* (20, 21). Indeed, these organisms seem to use PCD to regulate their population density (18, 19) or in response to different drugs (18, 20). Interestingly, it has been shown that lectin ConA stimulates apoptosis in trypanosomatids. During this ConA-induced death, the organism up-regulates the expression of certain mRNAs, indicating that trypanosomes actively participate in their suicide (20). Furthermore in *T. brucei*, five proteins related to mammalian caspases, known as metacaspases, have been identified, and one of these proteins (TbMCA4) induces cell death when expressed in yeast (21). Thus we can conclude that apoptotic processes also occur in kinetoplastidae, but that they are quite different from the mammalian mechanisms.

Dictyostelium discoideum

The life cycle of the unicellular slime mold *Dictyostelium discoideum* consists of a solitary growth phase followed by a social stage. During this phase, the individual cells aggregates to form a multicellular slug. Then, to construct the fruit body, the organism differentiates into two cell types, the viable spores and a stalk of dead cells. Thus about 20-25% of the cells die to form the stalk. This PCD shows several features of the mammalian apoptosis, such as the decrease of the mitochondrial transmembrane potential and the exposition of phosphatidyl serine residues at the plasma membrane (25, 26). The other characteristics of PCD in *Dictyostelium* are not clear yet. One study suggests that apoptosis in *Dictyostelium* results in DNA degradation that is mediated by a homologue of AIF in a caspase independent manner (25), whereas it is proposed in another study that caspase-3 activity increases in differentiating stalk cells without any DNA fragmentation (26). However both studies conclude that apoptosis has been well conserved during evolution.

Tetrahymena thermophila

In the ciliated protozoa *Tetrahymena thermophila*, PCD-like processes have also been observed in low density cell cultures, or after staurosporine induction (22). Furthermore, *Tetrahymena* shows a unique apoptosis-like “nuclear death” during conjugation. *Tetrahymena* contains one micronucleus that undergoes meiosis and is implicated in genetic exchange, and a somatic macronucleus that degenerates. This programmed “nuclear death” (PND) consists of chromatin condensation and DNA degradation. Interestingly caspase-like activities appear to play a role in this process (23). Moreover, PND in *Tetrahymena* also affects mitochondria. Some mitochondria are taken up by the autophagosome, the organelle responsible for macronucleus degradation and are disrupted in the process. This leads to release of mitochondrial factors, including an endonuclease showing similarities with mammalian Endonuclease G (24). So just as in *Dictyostelium*, PND in *Tetrahymena* shows similarities with the mammalian apoptosis.

Bacteria

Apoptosis has been mainly studied in eukaryotic organisms. But it has been recently reported that prokaryotes can also perform a kind of PCD. Indeed, the potential existence of apoptosis-like cell death has been suggested in several bacteria such as *E. coli*, *Staphylococcus aureus* or *Bacillus subtilis* (27-29). In these bacteria, PCD appears to take the form of cell autolysis. This process includes the self-digestion of the cell wall by peptidoglycan hydrolases that are also termed autolysins. Traditionally, autolysis has been thought to be the result of a misregulation of the normal peptidoglycan hydrolysis that is necessary for the cell wall building. However, more recent data suggest that the process can be considered as PCD (27). Interestingly, in some bacteria autolysis is also required for differentiation. *B. subtilis* for example needs it for the destruction of the mother cell and the release of the mature spore in order to complete sporulation. In some other cases, cells commit suicide to perform genetic exchange, meaning that the surviving cell will pick up the DNA from the lysed bacteria (27). Moreover, when a population of *E. coli* or *S. aureus* is exposed to antibiotics or other harmful conditions, they often perform autolysis which can be considered as an apoptotic manner to eliminate damaged cells (27, 28). Finally a recent study showed that in *B. subtilis* a high level of reactive oxygen species consecutive to shear stress leads to apoptosis-like cell death, which includes activation of a caspase-3-like protein and DNA fragmentation, two events characteristic of eukaryotic apoptosis (29). So PCD is not restricted to eukaryotic cells, but may also be present in evolutionary much older organisms such as bacteria.

c) <u>Unicellular organisms as model to study mammalian apoptosis</u>

As explained in point 1a), apoptosis is a very complicated process whose complete understanding could help a lot in treatment of several human diseases. Unfortunately studying PCD in mammalian cells is not trivial, because of the different pathways that exist and the many proteins that are involved in the process. The fact that apoptotic-like mechanisms have been revealed in unicellular organisms raises the question whether it is possible to use them for the study of mammalian apoptosis.

Yeasts have been already extensively used in this way (11, 30, 31). Indeed, heterologous expression of human Bax is sufficient to kill yeast cells. Most interestingly this death shows clear features of mammalian apoptosis. Moreover, co-expression with Bcl-x_L prevents Bax-induced apoptosis in yeast. Thus it was possible to use a human gene library to identify inhibitors of apoptosis (11, 30). Yeast can also be used to better understand the role of the mitochondria in PCD including the function of Bax and other members of the Bcl-2 family (31), and the role of mitochondrial fission proteins in the process (16). Human Bax is not the only protein whose expression induces PCD in yeast. Other pro-apoptotic factors, such as caspases or Apaf1, also lead to cell death (11, 30).

The kinetoplastid *Trypanosoma brucei* can also be used as a model to study mammalian apoptosis, as exemplified in chapter 1 of the result section. Indeed, as in yeast, *T. brucei* lacks most of the proteins involved in the classical mammalian apoptotic pathway (32). Moreover, inducible gene expression (or inducible RNAi) is well developed in this organism (33). And last but not least, unlike most other eukaryotes, trypanosomatids have a single mitochondrion only (34). This unique feature allows to obtain valid information about the temporal sides of apoptotic events. So *Trypanosoma brucei*, as yeast, is a nice potential model for the understanding of the mechanisms involved in mammalian apoptosis.

2. Mitochondrial Division

Mitochondria are complex double-membrane bound organelles found in nearly all eukaryotes, with their own genome and proteins synthesis machinery. Mitochondria carry out several important cellular functions, including ATP production through oxidative phosphorylation. Moreover, as presented in the part 1 of the introduction, these organelles are known to play a very important role in apoptosis. Depending on the organism and the cell type, mitochondria can occur in very different numbers and shapes, which among others might be determined by the energy needs of the corresponding cell. Interestingly, mitochondria cannot be synthesized *de novo*, meaning that pre-existing organelles must grow and divide during the cell cycle to be distributed to the daughter cells during cytokinesis. Furthermore, observations of living cells showed that mitochondria are very dynamic. They move around, change their shape, divide and fuse throughout the cell cycle (35). Thus these changes in morphology and distribution

can help to optimize mitochondrial function in response to changing intracellular needs and extracellular cues (36). The mitochondrial morphology depends mainly on the equilibrium between fission and fusion events. Loss of mitochondrial fission leads to excessive fusion, forming net-like mitochondria, whereas disturbed fusion results in fragmented organelles (Fig. 2) (36, 37).

The pathways of fission and fusion are well conserved during evolution. In the following chapter, I will mainly focus on mitochondrial division in yeast and mammals, and finally I will present some features of this pathway in other organisms such as nematodes, plants, algae and trypanosomes.

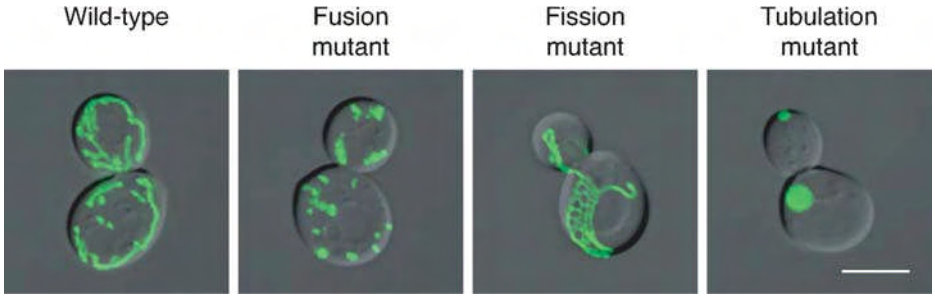


Figure 2: Mitochondrial morphology in the budding yeast *Saccharomyces cerevisiae*. In this organism, a third pathway, known as tubulation pathway, is involved to maintain the mitochondrial shape. Mitochondria are visualized by matrix-targeted GFP in different strains, respectively wt, *fzo1Δ*, *dnm1Δ* and *mmm1Δ*. Bar = 5μm (36).

a) <u>In yeast</u>

The budding yeast *Saccharomyces cerevisiae* is one of the favorite model systems to study mitochondrial dynamics. Indeed, the first proteins involved in mitochondrial distribution and morphology have been discovered in this organism through genetic screens (35, 36). In wild-type yeast, mitochondria form a branched tubular network located near the cell periphery, but mutations in several nuclear genes disturb this shape and result in specific morphology phenotypes (35-37). In addition to fusion and fission events, mitochondrial shape in yeast is also maintained by a pathway acting on tubulation (Fig.2) (36).

In budding yeast mitochondrial fission is regulated by Dnm1, a dynamin-like GTPase (36-39). As expected, Dnm1-defective yeast shows extensively fused mitochondria due to ongoing fusion. Interestingly however no other organelles are affected (38). Dnm1 contains an N-terminal GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED). Mutational analyses show that GTPase activity of Dnm1 is required for mitochondrial fission *in vivo* (36, 38). On the other hand, as in other dynamins, both middle and GED domains, are involved in protein-protein interactions (40). Biochemical analyses revealed that, whereas Dnm1 remains largely soluble in the cytosol, it can assemble into punctuated structures on the OMM. Interestingly, these clusters are mainly found at constricted sites on mitochondrial tubules that look like they are in the process of division (36, 37, 39). Moreover, it has been shown that Dnm1 interacts with itself to form rings *in vitro*, which could facilitate the fission of the mitochondria *in vivo*. As to confirm it, these extended Dnm1 spirals have diameters matching exactly the mitochondrial constrictions observed *in vivo* (41).

Dnm1 is not the only protein of the mitochondrial fission machinery in yeast. Another important component of this apparatus is Fis1. Contrary to Dnm1, Fis1 is a transmembrane protein equally distributed on the OMM (36, 37, 42), that has a TPR-fold (43). The protein is essential to recruit Dnm1 to mitochondrial fission sites, because the GTPase lacks a mitochondrial targeting sequence (37, 42). But direct binding of Fis1 and Dnm1 has never been shown. Instead, recent studies indicate that this interaction is mediated by Mdv1, a WD40 protein which acts as an adaptator between Fis1 and Dnm1 (44, 45). Mdv1 interacts directly with Fis1 through its N-terminal extension of unknown structure (NTE), whereas the C-terminal WD repeat mediates the binding to Dnm1. Finally the central coiled-coil domain of Mdv1 allows the formation of homo-oligomers (Fig. 3a) (45, 46). Mdv1 shows the same localization than Dnm1 and thus is also targeted to mitochondria through Fis1. Indeed, in absence of Fis1, neither Dnm1 nor Mdv1 are localized to mitochondria (36, 37, 45). In a recent model of mitochondrial fission, it is suggested that Fis1 first targets Mdv1 to division sites and that only then Mdv1 recruits Dnm1 (Fig. 3b) (36, 45). Finally, Caf4, another component of the mitochondrial fission complex, has been identified recently. Caf4 is also a WD40 protein showing similar structure and function than Mdv1 (Fig. 3a) (36, 46). Unlike in the cases of Dnm1, Fis1 and Mdv1, ablation of Caf4 does not affect mitochondrial morphology. However, in absence of both Mdv1 and Caf4, the mitochondrial fusion phenotype is stronger than the one observed in a Mdv1 mutant alone. This means that Mdv1 and Caf4 are redundant proteins, Mdv1 being the more important one (46).

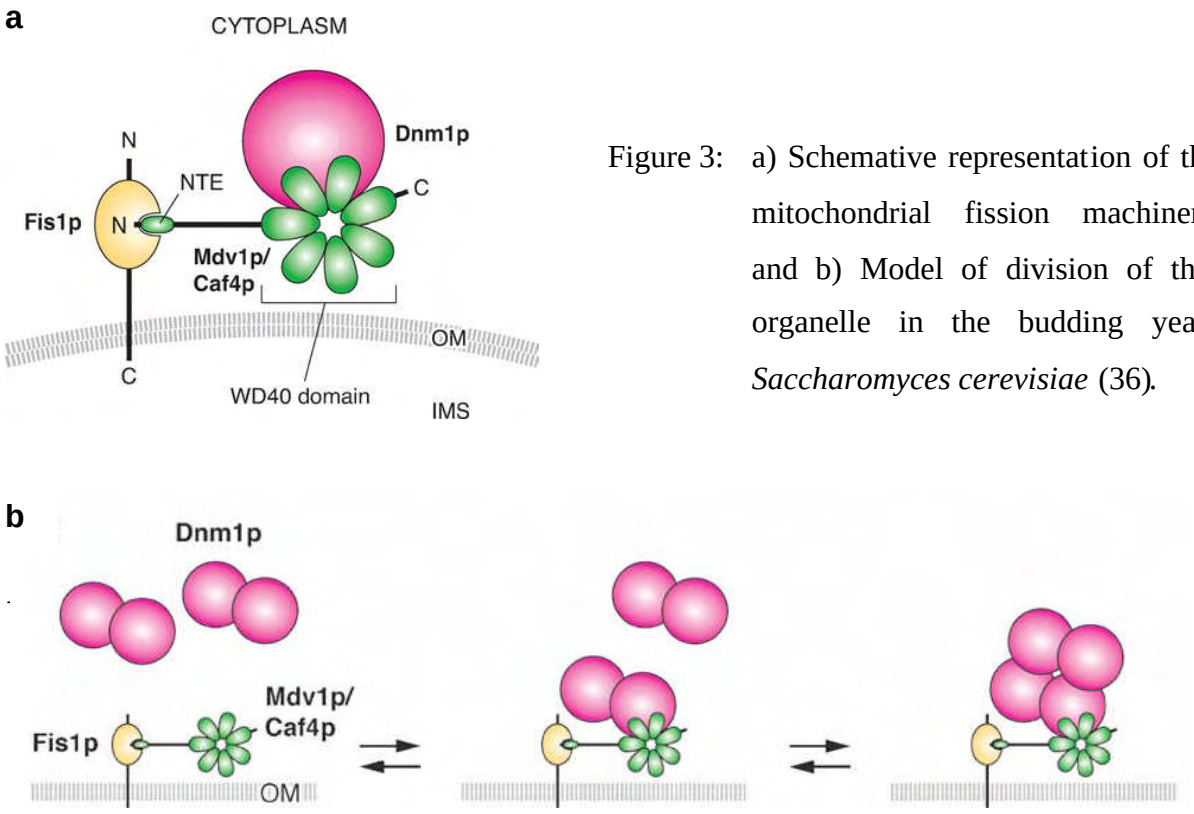


Figure 3: a) Schematic representation of the mitochondrial fission machinery and b) Model of division of this organelle in the budding yeast *Saccharomyces cerevisiae* (36).

b) <u>In mammalian cells</u>

The general mechanism of maintenance of the mitochondrial shape by opposing fission and fusion events is well conserved from yeast to mammals. However, some significant differences have been observed in the specific mechanisms. Some components are missing in the mammalian mitochondrial fission machinery. Indeed, whereas homologues of Dnm1 and Fis1 are present in most eukaryotes, Mdv1 and Caf4 seem to be restricted to yeast (36). Thus the two conserved components must somehow function differently than the yeast ones.

The Dnm1 homologue in mammalian cells is generally termed Drp1, but has been also called DLP1, DVLP1 or Dymple. As in yeast, Drp1 is mainly present in the cytosol and then translocates into a punctuated pattern on dividing mitochondria (47-49). The mammalian protein is also able to tubulate mitochondrial membrane and to form rings *in vitro* (50). The human homologue of Fis1 is highly similar to the yeast one in structure, localization and function (51-53). But the mechanism of recruitment of Drp1 to mitochondria is still unknown. It must clearly be different to the one in yeast because of the lack of Mdv1 and Caf4. A recent study has shown that Drp1 can directly bind to the TPR-repeats of Fis1, by doing so it is

recruited to mitochondria. Thus direct interaction of Drp1 with Fis1 not only determines the fission site, but is also required to achieve local Drp1 concentrations high enough for self assembly (54). However the interaction between Fis1 and Drp1 seems to be weak and transient (52, 54), and in line with this another study showed that inhibition of Fis1 does not affect Drp1 recruitment and localization. So Drp1 is not necessarily targeted to mitochondria in a Fis1-dependent manner (55).

Contrary to yeast, in mammalian cells Drp1 and Fis1 are both required for the division of peroxisomes, a mammalian organelle involved in hydrogen peroxide metabolism, β-oxidation of fatty acids and biosynthesis of ether phospholipids (56, 57). Like mitochondria, new peroxisomes form by division of preexisting ones (56). The functions of Drp1 and Fis1 look similar on mitochondria and peroxisomes, suggesting that the fission machinery of mitochondria and peroxisomes share common components. However they are not identical, since peroxisomal division requires peroxisome-specific proteins such as Pex11 that have no implication in mitochondrial fission (56, 57).

Interestingly, some additional factors are known to influence mitochondrial fission in mammals. Two studies showed a connection between the cytoskeleton and the recruitment of Drp1 to mitochondria (58, 59). The cytoskeleton is known to determine the subcellular localization of mitochondria. However its involvement in mitochondrial function was not expected (60). In the first study, the cytoplasmic dynein/dynactin complex mediating the minus-end-directed transport along microtubules has been shown to interact with Drp1. Moreover inhibition of dynein function resulted in fused mitochondria and translocation of Drp1 to cytosol, suggesting that dynein may control the recruitment of Drp1 to mitochondria (58). The second study showed that F-actin may also be implicated in this recruitment. However, unlike in the case of dynein, Drp1 is not translocated to cytosol when F-actin is disrupted, but it cannot be transferred anymore to mitochondria when fission is induced. This indicates that F-actin might be also required to facilitate the recruitment of Drp1 (59). Recently an other intramitochondrial protein termed MTP18 has been proposed to be a new essential component of the mitochondrial division apparatus. MTP18 bound to IMM is probably required to facilitate the fission step and thus contributes to the maintenance of mitochondrial morphology (61). Finally, human Drp1 has also been shown to interact with Sumo1 and Ubc9, two proteins involved in a posttranslational modification termed sumoylation and at least Sumo1 was shown to regulate mitochondrial fission (62).

Thus, even through there has been great progresses in the understanding of mitochondrial fission in mammals, many questions remain to be answered.

c) In other organisms

Whereas mitochondrial fission has been mainly studied in yeast and mammals, the process has also been investigated in other organisms such as nematodes (63-65), plants (66-72), algae (73, 74) and trypanosomes (75). In the following chapter, I will discuss the distinct features of mitochondrial fission that are observed in these organisms.

Caenorhabditis elegans

The genome of the nematode *C. elegans* encodes both Dnm1 and Fis1 homologues (www.wormbase.org). However, only the large GTPase, termed DRP-1, has been studied so far. Studies of this protein in *C. elegans* confirmed its role in mitochondrial fission. DRP-1 is essential for viability and mutants of the protein show a strong defect of mitochondrial segregation. Moreover, over-expression of the *C. elegans* DRP-1 results in excessive mitochondrial fragmentation. Interestingly, in *C. elegans* only the OMM seems to be affected by DRP-1 depletion, since severing of the IMM is still possible in the DRP-1 mutant (63). As in mammals, DRP-1 is also required for mitochondrial fragmentation during apoptosis (64). Furthermore a recent study showed that CED-9, the nematode Bcl-2 homologue, not only functions in regulating apoptosis, but also affects mitochondrial fission/fusion dynamics. Indeed, CED-9 expression in mammalian cells, just as it is the case for Bcl-2, induces fusion by a direct interaction with Mitofusin, a well-conserved component of the mitochondrial fusion machinery. On the other hand, the mechanisms of how PCD is controlled by CED-9 and Bcl-2 are distinct. Thus it is possible that the primordial function of the CED-9/Bcl-2 family may have been regulation of mitochondrial morphology (65).

Arabidopsis thaliana

The mechanism of mitochondrial division in higher plants has only recently been studied. But already the initial results that have been obtained show that there are major differences to the yeast and human systems. Thus, 16 dynamin-related proteins grouped in 6 subfamilies have been reported in *Arabidopsis thaliana* (66). The DRP3 gene family, consisting of DRP3A and DRP3B, also known as ADL2A and ADL2B, is most closely related to the standard

Dnm1/Drp1. Interestingly both DRP3A and DRP3B are colocalized and form a punctuated pattern on constricted mitochondria. Furthermore a mutation in any of the two proteins, leads to elongated mitochondria (67, 68). DRP3A has in addition been shown to control peroxisomal division, just as the mammalian Drp1 (69). But interestingly a recent study showed that DRP3B is not required for the apoptotic mitochondrial fragmentation in plants, suggesting that the mechanisms of programmed cell death is not the same in plants and animals (70). On the other hand, two additional dynamin-related proteins of another sub-family, DRP1C and DRP1E, actively function in mitochondrial fission. The two proteins show an identical speckled pattern, which is partially colocalized with mitochondria and DRP3B. Moreover, mutants for DRP1C and DRP1E show abnormal mitochondrial elongation, which can be counterbalanced by over-expression of the wild-type proteins. In summary these results suggest that these proteins are involved in mitochondrial fission (71). Finally, a homologue of Fis1 has recently been discovered in *Arabidopsis thaliana.* This protein termed BIGYIN, shows the same structure than its yeast and human homologues and is required for mitochondrial fission (72).

Cyanidioschyzon merolae

The red alga *Cyanidioschyzon merolae* is a primitive eukaryote containing a single chloroplast and a single rounded mitochondrion. The division of these two organelles is highly coordinated. Interestingly the genome of *C. merolae* encodes a homologue of Dnm1/Drp1, the mitochondrial fission factor in higher eukaryotes, as well as a homologue of bacterial FtsZ, that was shown to be required for mitochondrial division is some lower eukaryotes (73). In *C. merolae* mitochondrial division appears to be organized in three distinct phases. First FtsZ forms a ring in the matrix and determines the division site. Then the mitochondrion-dividing ring, an electron-dense structure, constricts the mitochondrion and finally Dnm1 severs the membranes (73). Interestingly mitochondrial division in *C. merolae* is cell-cycle dependent, showing changes in the expression or localization of fission factors at specific stages of the cycle. Moreover, microtubules were shown to be involved in mitochondria segregation, but have no influence on the division itself (74), contrary to what is observed in mammalian cells (58, 59). It is generally assumed that in higher eukaryotes Dnm1/Drp1 has replaced FtsZ. The observation that Drp1 severs only the OMM in *C. elegans* (63) seems to confirm this hypothesis. However the fact that no FtsZ homologue is found in *C. elegans* raised the question of how the IMM is divided.

Trypanosoma brucei

The parasitic protozoon *Trypanosoma brucei* is a nice model to study mitochondrial division since, as *C. merolae*, it contains a single mitochondrion only that has a network-like structure (34). Interestingly, the genome of *T. brucei* encodes only a single dynamin. Functional analysis of this dynamin constitutes part 2 of the result section of this thesis. Furthermore, a preliminary analysis of the *T. brucei* Fis1 homologue is presented in part 3 of the result section.

3. Cell Cycle in *Trypanosoma brucei*

A dividing cell undergoes a succession of well-organized and defined events known as the cell cycle. This cycle consists of four different phases each playing a specific role. During the first phase, termed G_1, the cell grows until it reaches a specific size and prepares its DNA to be replicated. Then the cell enters the S-phase, where its DNA is duplicated. The next phase, termed G_2, consists of further cell growth and preparations for the cell division. These three first phases form the interphase, which time-wise accounts for the main part of the cell cycle. Then the cell enters in the nuclear division step, known as mitosis. According to the condensation stage and position of the chromosomes, mitosis can be subdivided into four stages: prophase (chromosomes condensation), metaphase (chromosomes binding to mitotic spindle and alignment of them on the metaphase plate), anaphase (separation of the two sets of chromosomes) and telophase (reformation of the nuclear envelope and DNA decondensation). Finally, the cleavage furrow appears and the cell proceeds to cytokinesis, the separation of the cytoplasmic compartments that ends in the formation of two daughter cells. After division, the two cells are back in G_1 phase and the cell cycle is completed (Fig 4) (76).

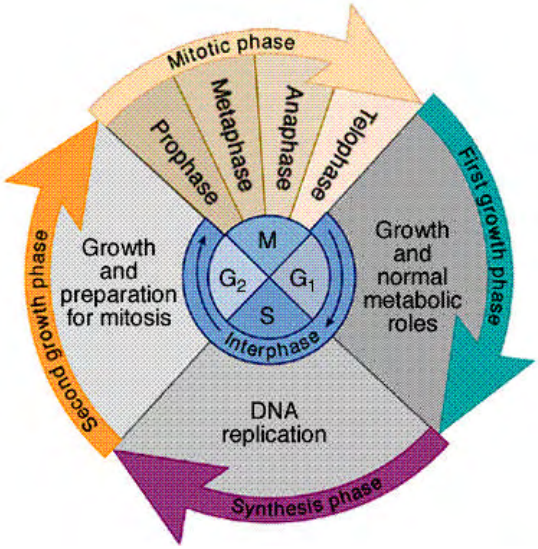


Fig. 4: Schematic representation of the cell cycle in an animal cell. The duration of mitosis in relation to the other phases is exaggerated in this diagram. (http://www.biologycorner.com/resources/cell_cycle.jpg)

The cell cycle must be finely regulated. Two major families of proteins are implicated in the control of cell cycle progression: the cyclins and the cyclin-dependant kinases (CDKs). The concentration of cyclins cyclically fluctuates during the cell cycle, whereas CDKs are present in similar amounts throughout the cycle, but in different activation stages. Indeed, only the binding of a specific cyclin to its corresponding kinase allows its activation and the subsequent phosphorylations. These phosphorylations will then activate or inactivate target proteins in order to orchestrate coordinated entry into the next phase of the cell cycle (76).

Because misregulation of the cell cycle can have dramatic consequences, the cell developed a molecular system of checkpoints. Thus cell cycle progression is interrupted if a number of key events have not properly occurred or if the DNA has been damaged during replication. This ensures that the cell only divides when it has completed all required steps to guarantee the production of two healthy daughter cells. In the case the checkpoints do not function properly, the division of the cells is uncontrolled and this often results in cancer.

The cell cycle of *Trypanosoma brucei* shows some unique features that are discussed in the following chapter. I will first describe the mechanisms of duplication of the different single organelles in this unicellular organism. Then I will review the main factors that have been implicated in the regulation of the cell cycle. Finally I will present non-cyclic proteins that are known to influence the cell cycle progression in *T. brucei.*

a) Duplication of single-copy organelles in the procyclic *T. brucei*

The African parasitic protozoon *Trypanosoma brucei* possesses several organelles in single copy, all of which have to be duplicated during the cell cycle. The mechanisms of division of these organelles are not completely understood yet. In the next few paragraphs I will review the main advances that have been made in recent years regarding these duplication processes.

Kinetoplast DNA

A very specific characteristic of *Kinetoplastidae* is their mitochondrial DNA. Whereas the mitochondrial DNA is generally distributed all over the matrix, the mitochondrial genome of *Kinetoplastidae* is contained in a discrete structure termed the kinetoplast, which is always located in the region of the mitochondrion that is near the base of the flagellum. The

kinetoplast DNA, or kDNA, contains two types of circular DNA molecules termed minicircles and maxicircles that form a highly concatenated network. Minicircles occur as a heterogeneous population of about 10’000 molecules of 1kb in length and encode guide RNAs that act in RNA editing. The maxicircle population consists of 50 homogenous copies. They are approx. 22kb in size and encode mitochondrial proteins (77).

Because of the structural unity of the kinetoplast, the mitochondrial DNA does not divide continuously as in other organisms, but shows a cycle of division that is similar to the nuclear one. Thus, replication of mitochondrial DNA occurs only once at a precise time of the cell cycle. Subsequently similar to the nuclei during mitosis the kDNAs segregate. Interestingly, the duplication and segregation of nuclei and kDNA are coordinated, but do not simultaneously. Replication of the mitochondrial DNA is always initiated before the nuclear S phase, and separation of the kDNA invariably occurs before mitosis (Fig. 5a) (78). Thus, counting the numbers of kDNAs and nuclei on a Dapi-stained slide allows to define three cell cycle stages in *T. brucei*: one kinetoplast and one nucleus (1K1N) corresponds to the nuclear G_1-S phases; two kDNAs and one nucleus (2K1N) corresponds to the G_2 stage and two kinetoplasts and two nuclei (2K2N) represents the mitotic and post-mitotic phases of the cell cycle (Fig. 5b) (78).

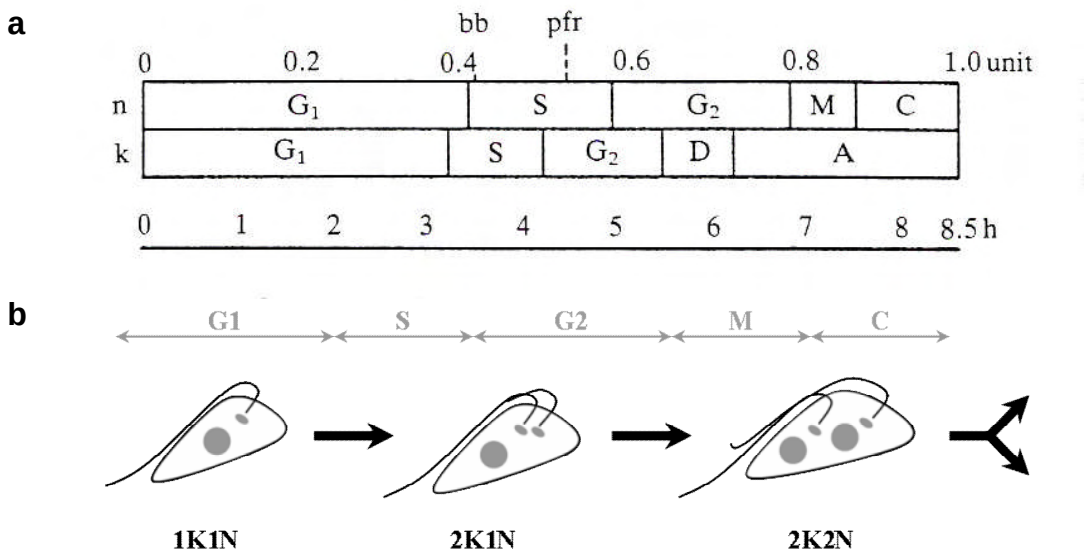


Fig. 5: Schematic representations of the cell cycle of *Trypanosoma brucei*. a) Durations of nuclear (n) and kinetoplast DNA (k) replication cycles represented in a linear map, bb = initiation of basal body duplication, pfr = initiation of paraflagellar rod synthesis (78). b) Schematic draw of the three visual cell cycle stages.

A structure known as the tripartite attachment complex (TAC) links the kDNA and the basal body (BB), which represents the base of the flagellum (77, 79). The TAC is composed of three different elements: the exclusion zone filaments, which link the proximal end of the BB to the adjacent OMM, the unilateral filaments, which are present only on the side of the kDNA facing the basal body and that link the kinetoplast to the IMM, and the differentiated mitochondrial membranes, showing linear profiles without cristae. The TAC duplicates together with the basal bodies during the S phase of the kDNA (Fig. 6) (80). Thus the segregation of the kDNA depends on the duplication of the BB and the flagellum.

Basal body, Flagellum and Flagellar Pocket

Wild-type G_1 cells have two basal bodies: a mature one at the base of the flagellum, and an immature one, which has not yet formed its own flagellum. Progressing through the cell cycle, the immature BB becomes mature and initiates the growth of a new flagellum. This process is accompanied by the formation of two new immature basal bodies (Fig. 6) (81, 82). Inhibition of BB segregation has been shown to block cytokinesis, confirming the essential role of these structures for the cell cycle progression (83). Recent studies showed that duplication of basal body is highly regulated. The conserved coiled-coil protein TbLRTP and the NIMA-related kinase TbNRKC, both components of the basal bodies, were shown to be implicated in the separation of the basal bodies in antagonistic ways: TbLRTP suppresses BB replication, whereas TbNRKC promotes it (81, 84).

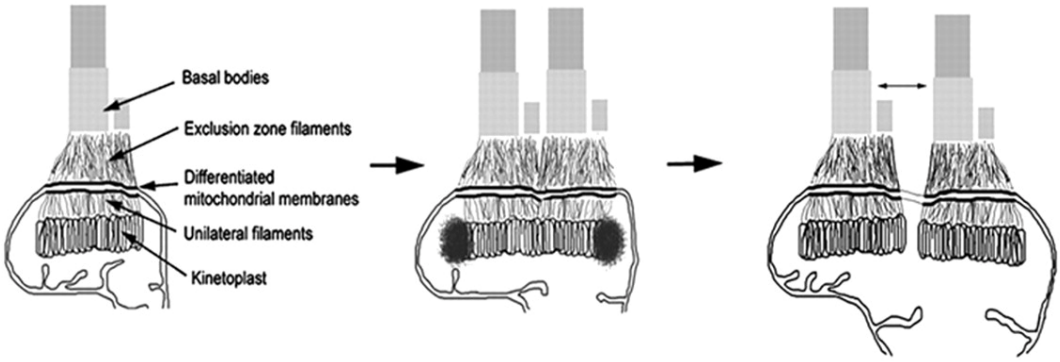


Fig. 6: Schematic representation of the TAC complex and its replication in trypanosomes (80).

Once the basal bodies are duplicated, the new flagellum can start growing. As the new flagellum elongates, its distal tip remains in constant contact with the old flagellum. This tethering is mediated by the flagella connector, a discrete transmembrane junction that is formed early during flagellar extension and removed at the end of cytokinesis (85, 86). The

flagellum is also attached along the length of the cell body. A cytoskeletal structure, termed the flagellar attachment zone (FAZ), is found in the cytoplasm adjacent to flagellum. Two structures form the FAZ, a set of four microtubules and an electron-dense filament. Interestingly the FAZ has been shown to determine the direction of cleavage and thus is essential for cytokinesis (85). This is supported by the observation that ablation of the protein FLA1, which is responsible for the attachment of the flagellum to the cell body, leads to a cytokinesis defect, but has no influence on kDNA segregation (87).

The flagellum emerges from the flagellar pocket (FP) of the cell body. This portion of the plasma membrane lacks the subpellicular microtubules, and therefore allows vesicular traffic. Thus, endocytosis and exocytosis are restricted to this small fraction of the plasma membrane (77, 82). Only when the flagellum exits the FP, the formation of the paraflagellar rod (PFR), an extra-axonemal structure, is initiated.

Visualizing all these structural components, the cell cycle of *T. brucei* can be split in up to ten different stages. Each of these stages is defined by a specific development stage of the basal bodies, the kDNA, the flagellum, and the nucleus (88).

Golgi apparatus

The Golgi apparatus is an essential organelle of the eukaryotic secretory system, required for the modification and sorting of newly synthesized proteins. Mammalian cells contain several hundreds of Golgi structures, consisting of stacks of flattened cisternae. The multicopy nature of the mammalian Golgi makes the study of its division difficult. Interestingly, *Trypanosoma brucei* contains only a single Golgi stack. Recent studies showed that during cell division its new Golgi is in principle formed *de novo*, but uses membrane components of the old one. The trypanosome Golgi is closely linked to basal body and appearance of the new Golgi closely follows BB duplication. Furthermore when basal bodies replication is disrupted, the Golgi duplication also is also affected (89). A recently discovered bilobed structure was shown to determine the site for the assembly of the new Golgi apparatus. The old Golgi is adjacent to the anterior lobe, whereas the new one appears to be associated with the posterior lobe. Finally when the new Golgi grows and separates from the old one, this bilobed structure duplicates too. Interestingly, one component of the bilobed structure is Centrin2. Centrins are highly conserved Ca^{2+}-binding proteins present in centrosomes. In *T. brucei* the structure homologous to the centrosomes are the basal bodies (90).

Mitochondrion

The second and third sections of the results reveal some aspects of the mechanism of division of the single mitochondrion of *Trypanosoma brucei*. In part two of the results, we show that mitochondrial division is essential for completion of cytokinesis, confirming the importance of the division of the single-copy organelles for the cell cycle progression.

b) Regulation of cell cycle progression

The cell cycle is highly regulated to guarantee the formation of normal daughter cells. In yeast, at least three checkpoints have been determined, that for the most part are conserved through evolution. Thus DNA synthesis is only initiated if the DNA is not damaged. Another checkpoint verifies that the mitotic spindle is correctly assembled before the initiation of mitosis. Finally mitosis must correctly be completed before the initiation of cytokinesis. Interestingly *Trypanosoma brucei* lacks some of these checkpoints. Treatment of *T. brucei* with different antimicrotubule drugs leads to the formation of unviable cells with one kinetoplast DNA but lacking nucleus (1K0N). These cells, termed zoids, are the result of continuing cytokinesis in the absence of mitosis (83). Conversely, defective cytokinesis leads to accumulation of multinucleated cells, showing that mitosis can go on even if the cell is no more able to divide. A very similar phenotype is observed when *T. brucei* is treated with okadaic acid, a protein phosphatase inhibitor (91).

Cyclins and CDKs

As described above, cell cycle progression is regulated through cyclins and CDKs. Eight cyclins homologues (CYC2-9) have been identified in *T. brucei* (92). The PHO80-like cyclin CYC2 is required for entry in S-phase (93, 94), whereas the B-type cyclin CYC6 is essential for the G2/M transition (93, 95). Thus, inhibition of CYC2 leads to accumulation of 1K1N cells (93, 94) and disruption of CYC6 results in zoid formation (95). Two other cyclins, CYC4 and CYC8, apparently involved in the initiation of S-phase and mitosis respectively, are not essential but influence the growth rate. Their depletion slows down cell division and shows partial G_1 arrest and an accumulation of zoids. Finally, disruption of CYC3, CYC5 and CYC7 has no effect on growth or cell cycle progression (93). CYC9 has not been studied yet.

The genome of *T. brucei* furthermore encodes five CDKs homologues (CRK1-4 and CRK6). CRK1 has been shown to control the G_1/S phase transition, whereas CRK3 is involved in G_2/M transition. As expected CRK3 has been shown to bind CYC6 (96), but also interacts with CYC2 (97). The other CRKs play only minor roles in cell cycle regulation (96). Thus, even when they are down-regulated in combination with CRK1 or CRK3, they do not enhance the phenotypes that ablation of CRK1 or CRK3 causes by themselves (98, 99). However the double knock-down of CRK1 and CRK2 shows an additional phenotype. Some of the G_1 arrested cells show multiple branched posterior ends that are not seen in the CRK1 knock down alone. This suggests a potential role of CRK2 in the control of the growth of the posterior microtubules (98).

Another interesting point is that disruption of CYC6 or CRK3 function revealed differences between the cell cycle regulation of procyclic and bloodstream forms of *T. brucei*. In both life cycle stages, the cyclin and the CDK regulate the G_2/M phase transition, however unlike procyclic cells the bloodstream forms are not able to enter cytokinesis if mitosis is blocked, and thus do not form zoids (99). Analysis of a double knock-down of CRK1 and CRK2 shows further differences between the cell cycle regulation of procyclic and bloodstream forms. In both life cycle stages there is an accumulation of G_1 cells and about 50% of these cells are incapable of DNA synthesis in the procyclic form. In bloodstream form DNA synthesis is not affected (100). Thus the mechanisms of cell cycle regulation are not identical in the different life cycle stages of *T. brucei.*

c) Other proteins implicated in cell cycle regulation

Additional proteins that are not related to cyclins or CDKs have been shown to play roles in controlling the cell cycle progression. These are often proteins implicated in posttranslational modifications and gene silencing. Thus, these proteins generally act indirectly on the cell cycle progression by controlling the activation or deactivation of cyclins and CDKs.

The ubiquitin-proteasome pathway is an example of cyclin-independent way of regulating cell cycle progression. Ubiquitination of short-lived and misfolded proteins by different enzymes targets them to proteasome for degradation. Indeed in *Trypanosoma brucei*, either depletion of the anaphase-promoting complex also known as cyclosome (referred as APC/C), which is

involved in the ubiquitination pathway (101), and the inhibition of the proteasome subunits (102, 103) leads to a G_2/M arrest. This means that the ubiquitin-proteasome pathway is required for entry in mitosis. Interestingly the consequences of disruption of the APC/C subunits are different in procyclic and bloodstream forms. It results in an arrest in the metaphase in procyclic forms, but in a block in late anaphase in the bloodstream stage (101). A difference was also observed when the proteasome itself is disrupted, since unlike in procyclic cells, in bloodstream forms this affects not only the G_2/M transition, but also the initiation of the S-phase, confirming that cell cycle regulation is different in the two to life cycle stages (103).

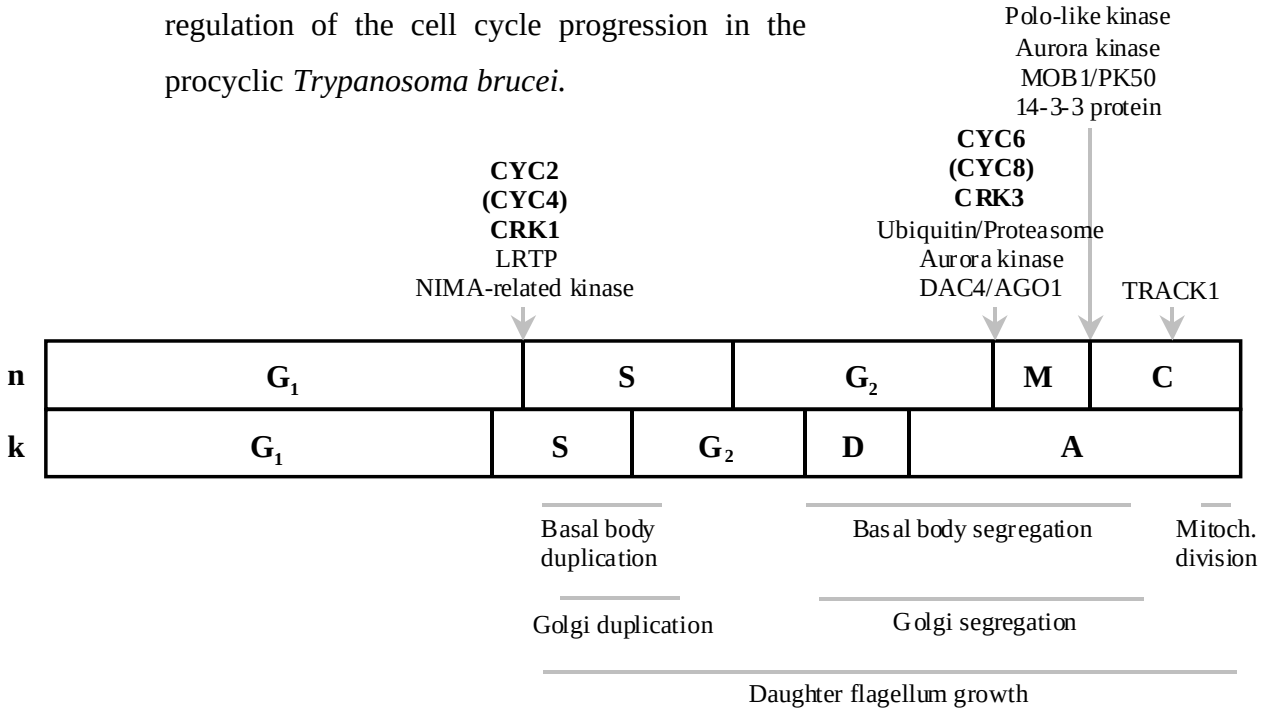
In addition to CDKs, there are many other kinases that are involved in cell cycle regulation, such as polo-like kinases (Plks). These enzymes are known in many different organisms to control both mitosis and cytokinesis. In *T. brucei,* knock-down of the single Plk gene leads to multinucleated cells, whereas its over-expression results in the formation of zoids. These results suggest that in *T. brucei* Plk is required for the initiation of cytokinesis but not for the control of mitosis (104). On the other hand, a *T. brucei* homologue of an aurora kinase (TbAUK1) is implicated in both mitotic spindle formation and cytokinesis, since its depletion leads to the accumulation of 2K1N cells (105). Furthermore, TbPK50, a functional homologue of Orb6, a yeast kinase involved in cell morphology and cell cycle progression, is present in *T. brucei* (106). Whereas its direct implication in regulation of the cell cycle has not been demonstrated yet, inhibition of MOB1, a protein known to interact with TbPK50, leads to a cytokinesis defect. In this case too, differences between procyclic and bloodstream forms have been demonstrated (107). Finally, inhibition of a number of different kinases results in unspecific aberrant kinetoplasts and nuclei configurations without clear accumulations at any one cell cycle stages. Examples for these include a mitogen-activated protein kinase (MAPK2) (108) and a kinase related to MAPKs (TbECK1) (109). Possible explanations for these phenotypes are that the signaling cascades activated by these proteins are required for more than one checkpoint (108, 109).

Another way of regulating cell cycle progression is by gene silencing at the chromatin level. Thus, inhibition of a trypanosomal histone deacetylase (DAC4), a protein family known to be involved in heterochromatin formation, results in a delayed entry into mitosis, and a subsequent partial accumulation of 2K1N cells (110). An even stronger effect is observed when TbAGO1, an Argonaute protein required for RNA interference (RNAi), is ablated

(111). As for histone deacetylases, RNAi is implicated in heterochromatin formation. Thus, an explanation for the observed phenotypes would be that since chromosomes are condensed into heterochromatin at the centromere during prophase, deficiencies in this condensation, due to either the lack of DAC activity or deficiencies in the RNAi pathway, leads to defective chromosome segregation (110).

Finally, other proteins that are not kinases were shown to influence cell cycle progression. A 14-3-3 protein for example is required for cytokinesis, probably because its potential binding partner, protein phosphatase 1 (PP1), might regulate the phosphorylation level of microtubules. This in turn could influence interactions between microtubules and microtubules binding proteins, resulting in an aberrant cytokinesis (112). The nuclear scaffold protein TRACK1, the trypanosome homologue of the receptor for activated C-kinase 1 (RACK1), is also required for cytokinesis. Interestingly, it is required for cytokinesis initiation in bloodstream forms, whereas in procyclic form it is only required for completion of cytokinesis (113). Finally, synthesis of the bloodstream-specific variant surface glycoprotein (VSG) acts as a checkpoint for initiation of cytokinesis. Thus inhibition of VSG synthesisleads to 2K2N accumulation and blocks any subsequent mitosis. These results show that VSG-synthesis has to be coordinated with cell division (114).

Fig.7: Summary of the proteins implicated in the regulation of the cell cycle progression in the procyclic *Trypanosoma brucei.*



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II. RESULTS 1

Temporal dissection of Bax-induced events leading to fission of the single mitochondrion in *Trypanosoma brucei*

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Temporal dissection of Bax-induced events leading to fission of the single mitochondrion in *Trypanosoma brucei*

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The protozoan *Trypanosoma brucei* has a single mitochondrion and lacks an apoptotic machinery. Here we show that expression of the proapoptotic protein Bax in *T. brucei* causes the release of cytochrome *c*, the depolarization of the mitochondrial membrane potential and mitochondrial fission. However, in contrast to mammalian cells, the three events are temporally well separated. The release of cytochrome *c* from the intermembrane space precedes mitochondrial fission, showing that it does not depend on mitochondrial fragmentation. Furthermore, halting Bax expression allows some cells to recover even after mitochondrial fission, the last recorded event, went to completion, indicating that all three Bax-induced events are, in principle, reversible.

Keywords: apoptosis; cytochrome *c*; membrane potential

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INTRODUCTION

The main components of the apopotic death machinery include the pro- and anti-apoptotic members of the Bcl-2 protein family as well as caspases. Mitochondria are central players in apoptosis, as activation of the caspase cascade is often initiated by the release of cytochrome *c* and other intermembrane space components (Desagher & Martinou, 2000; Ferri & Kroemer, 2001; Newmeyer & Ferguson-Miller, 2003). In addition to permeabilization of the outer membrane, a loss of the membrane potential and, in some cases, changes in mitochondrial morphology such as fission are observed (Desagher & Martinou, 2000; Frank *et al*, 2001; Karbowski *et al*, 2002). All effects are initiated by pro-apoptotic proteins such as Bax, which directly interact with mitochondria. However, by which molecular mechanisms they exert their effects and in which order is still unclear.

Some forms of programmed cell death have been described in the parasitic protozoan *Trypanosoma brucei*; however, they are clearly different from the classical apoptosis observed in mammalian cells (Welburn & Murphy, 1998; Ameisen, 2002; Debrabant *et al*, 2003). In agreement with this, a survey of the available *T. brucei* genome did not reveal any homologues for caspases or for members of the Bcl-2 protein family. Genes encoding metacaspases, on the other hand, which show only a limited similarity to bona fide caspases, were found. However, it is unclear at present whether trypanosomal metacaspases have protease activity and whether they have a role in programmed cell death (Szallies *et al*, 2002). Interestingly, *T. brucei*, unlike any other eukaryote, has a single continuous mitochondrion throughout its life and cell cycle (Simpson & Kretzer, 1997; Tyler *et al*, 2001). Its genome is exclusively localized at a precise position in the posterior region of the organelle. Thus, *T. brucei* provides an excellent system to study the effects of pro-apoptotic proteins on the single mitochondrion level in the absence of apoptotic death effectors.

RESULTS AND DISCUSSION

Bax-induced effects on mitochondrial energy metabolism

To take advantage of its unique mitochondrial biology, we have established a transgenic *T. brucei* cell line allowing tetracycline-inducible expression of human Bax. Similar to yeast (Greenhalf *et al*, 1996; Harris *et al*, 2000), expression of Bax inhibits growth and will eventually kill the cells. Is the observed death of physiological significance in that it mirrors the events in apoptotic mammalian cells? To address this question, we have established a trypanosomal cell line that, upon addition of tetracycline, expresses not only Bax but also the Bax antagonist Bcl-x_L. Fig 1 shows that, similar to mammalian cells, Bcl-x_L expression inactivates Bax. The growth rate of the Bax/Bcl-x_L-expressing cell

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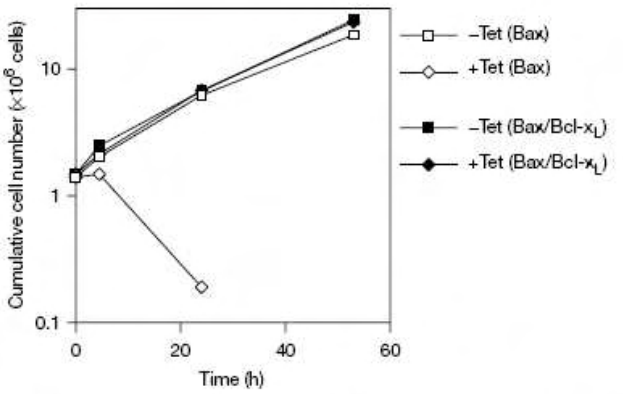

Fig 1 | Effect of Bax expression and Bax/Bcl-x_L coexpression on the growth of *T. brucei*. Growth curves of *T. brucei* cell lines transformed with the human Bax cDNA (Bax) alone or with both the human Bax and Bcl-x_L cDNAs (Bax/Bcl-x_L) together, respectively. Cumulative cell numbers for growth in the absence (−Tet) and presence (+Tet) of tetracycline are shown.

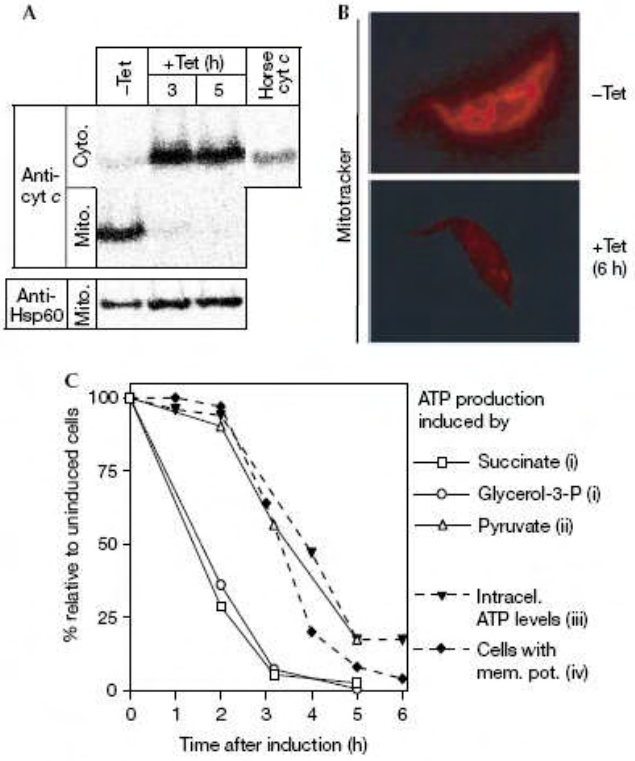

Fig 2 | Bax-induced changes of cytochrome *c* localization and of mitochondrial energy metabolism. (**A**) A transgenic *T. brucei* cell line allowing tetracycline-inducible expression of human Bax was analysed for the release of cytochrome *c*. Using immunoblots, 50 μg each of cytosolic extract (upper panel) and of isotonically isolated gradient-purified mitochondria (middle panel; Hauser *et al*, 1996) from uninduced cells (−Tet) and from cells expressing Bax for 3 or 5 h (+Tet (h) 3 or 5), respectively, were analysed for cytochrome *c* content. All mitochondrial fractions were also analysed for the presence of Hsp60 (lowest panel). (**B**) Mitotracker staining of an uninduced cell with an intact mitochondrial membrane potential (−Tet) and a cell expressing Bax for 6 h that lacks a membrane potential (+Tet, 6 h). (**C**) Time course of Bax-induced changes in mitochondrial energy metabolism. Four parameters were measured at the indicated time points after induction of Bax expression: (i) *in organello* ATP production in response to succinate and glycerol-3-phosphate to monitor oxidative phosphorylation; (ii) *in organello* ATP production in response to pyruvate to measure mitochondrial substrate level phosphorylation (van Hellemond *et al*, 1998; Bochud-Allemann & Schneider, 2002); (iii) intracellular ATP levels (ATP production and ATP content are indicated relative to uninduced cells); and (iv) the presence or absence of a mitochondrial membrane potential in individual cells ($n = 150–300$) as determined by Mitotracker staining (expressed as the percentage of the total population that retains a membrane potential).

line is the same as that of uninduced cells, even though a small subpopulation of cells (<1%) still shows a Bax-induced change in mitochondrial morphology (not shown). The Bax protein that is expressed in *T. brucei* is therefore correctly folded and able to interact with its regulators. During the first 12 h after induction of Bax expression, the cell density remains constant and no change in cell morphology is observed. During that period, it is possible to measure the effects of Bax that precede cell death.

The immunoblot in Fig 2A shows that essentially all cytochrome *c* is released within the first 3 h after induction of Bax expression. *In organello* ATP production assays, induced by the respiratory substrates succinate and glycerol-3-phosphate, were used to quantify oxidative phosphorylation (Allemann & Schneider, 2000; Bochud-Allemann & Schneider, 2002). The results in Fig 2C show that, concomitant with the release of cytochrome *c*, a loss of oxidative phosphorylation is observed, suggesting that at early time points Bax permeabilizes the mitochondrial outer membrane and that the release of cytochrome *c* causes the loss of oxidative phosphorylation.

We next investigated the effect of Bax on the mitochondrial membrane potential by using Mitotracker, a membrane-potential-sensitive dye. The Mitotracker staining revealed two types of cells: cells having an intact membrane potential showing the single network-like mitochondrion and cells with a depolarized potential showing a weaker uniform staining (Fig 2B). Fig 2C shows that the loss of oxidative phosphorylation precedes the collapse of the membrane potential by approximately 2 h. These observations are in agreement with reports showing that apoptotic mammalian cells can maintain the mitochondrial membrane potential for a limited time even after cytochrome *c* has been released (Mootha *et al*, 2001; Waterhouse *et al*, 2001). However, unlike as suggested for mammalian systems (Ricci & Gottlieb, 2003), the loss of the membrane potential observed in trypanosomal mitochondria cannot be caused by caspases.

Fig 2C shows furthermore that, most likely as a consequence of the depolarization of the membrane potential, a synchronous loss of the pyruvate-induced mitochondrial substrate level phosphorylation (Bochud-Allemann & Schneider, 2002), a drop of the total cellular ATP content and a decline of flagellar motility occur (not shown).

Bax-induced mitochondrial fission

To monitor directly Bax expression and to investigate its effects on mitochondrial morphology, we performed immunofluorescence

using antibodies directed against Bax and the mitochondrial matrix heat shock protein 60 (Hsp60; Fig 3A). Bax was only detected in induced cells and showed a punctate pattern whose kinetics of appearance coincided with the loss of the membrane potential (Fig 3A,C). The Hsp60 staining shows that, at early time points, cells with detectable Bax exhibit a normal mitochondrial morphology. However, approximately 2.5 h later, the single mitochondrion of uninduced cells becomes fragmented into 7–9 distinct spherical compartments (Fig 3A). The punctate Bax staining colocalizes with the mitochondrial fragments; however, it generally does not completely overlap with mitochondrial vesicles but appears to be concentrated in dots localized at their periphery. Few mitochondrial fragments lacking Bax as well as few Bax-containing structures devoid of Hsp60 are also seen. The highly condensed mitochondrial genome of *T. brucei* is easily detected by DAPI fluorescence and always colocalizes with a mitochondrial fragment.

To confirm that the observed mitochondrial fragmentation is a specific consequence of Bax expression and not just associated with any kind of cell death, we made use of an RNA interference (RNAi) cell line ablated for succinyl-CoA synthetase, a mitochondrial enzyme essential for survival of insect-stage *T. brucei* (Bochud-Allemann & Schneider, 2002). The right panel of Fig 3A shows that the mitochondrial morphology in these cells remains normal at a time point when the cells are already irreversibly committed to death.

Electron micrographs show that the outer and the inner membranes can clearly be distinguished in the fragmented mitochondria of induced cells, indicating that Bax expression does not cause a global rupture of the mitochondrial outer membrane (Fig 3D). After 8 h of Bax expression, the entire cell

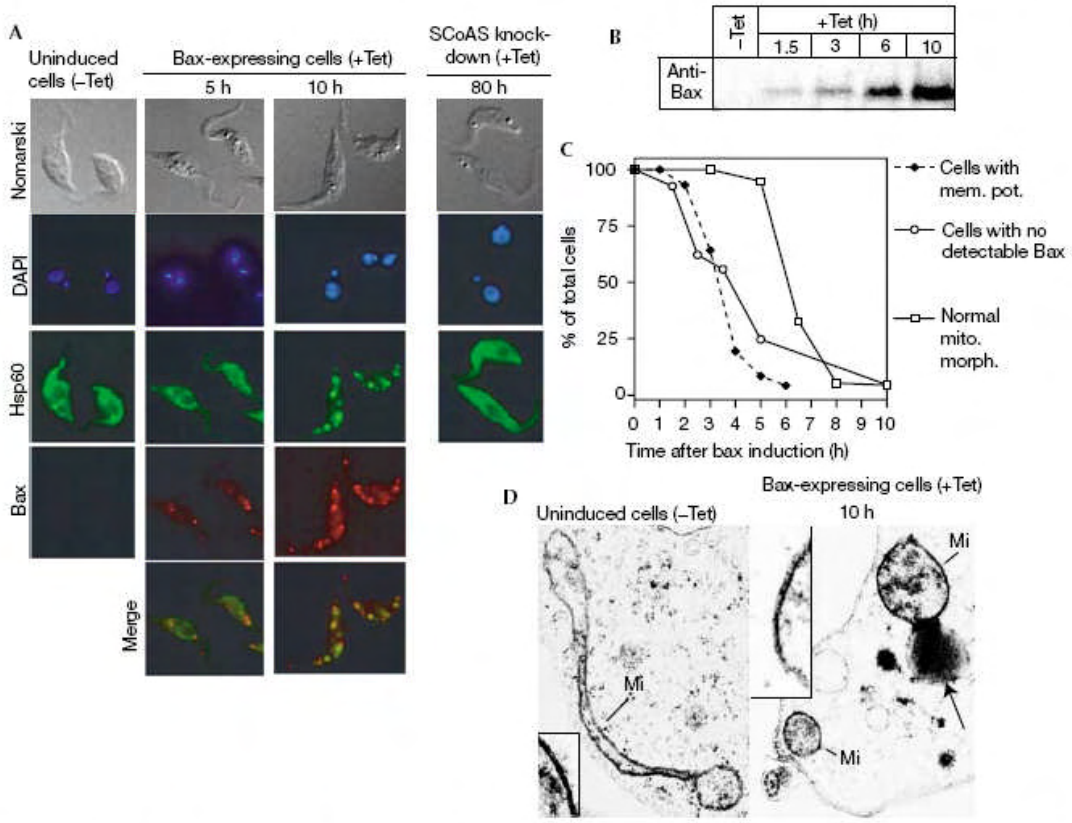

Fig 3 | Bax-induced changes of mitochondrial morphology. (**A**) Left panel: the *T. brucei* cell line transformed with the human Bax cDNA was analysed for Bax expression and mitochondrial morphology using double immunofluorescence with Hsp60 and Bax antisera, respectively. Left column: uninduced cells (−Tet); middle and right columns: Bax-expressing cells induced (+Tet) for 5 and 10 h, respectively. Right panel: Hsp60 staining of an RNA interference (RNAi) cell line downregulated for the essential mitochondrial enzyme succinyl-CoA synthetase (SCoAS) 80 h after induction of RNAi (Bochud-Allemann & Schneider, 2002). At this time, the culture is dying but no mitochondrial fission is observed. The length of a *T. brucei* cell is approximately 20 μm. (**B**) Time course of Bax detection on immunoblots. (**C**) Time course of the appearance of the punctate Bax staining and mitochondrial fission. Double immunofluorescence of cells from a culture induced for Bax expression was used to score the presence of the punctate Bax staining and the occurrence of mitochondrial fission. The relative proportions of each cell culture ($n = 150$–300 cells), which at the indicated times after induction of Bax expression still show the phenotypes of uninduced cells, namely absence of Bax and a single mitochondrion, are indicated on the *y*-axis. For comparison, the disappearance of the mitochondrial membrane potential (same curve as shown in Fig 2C) is shown as well. (**D**) Electron micrographs of uninduced cells and cells grown in the presence of tetracycline for 10 h. The double mitochondrial membranes are shown in the insets. Electron-dense material attached to mitochondrial vesicles (Mi) seen in induced cells is indicated by the arrow.

culture essentially only consists of cells that have a fragmented mitochondrion (Fig 3C). However, a low number of cells (approximately 5%) still show normal mitochondrial morphology even though most of them express Bax.

Mitochondrial fission observed during mammalian apoptosis is due to recruitment of dynamin-related protein 1 (Drp1) and Mfn2, two proteins that are involved in fission of the mitochondrial outer membrane in normal cells (Frank *et al*, 2001; Karbowski *et al*, 2002). Furthermore, overexpression of a dominant-negative mutant of Drp1 was shown to inhibit the loss of the membrane potential as well as the release of cytochrome *c*, suggesting that mitochondrial fission is required for both processes (Frank *et al*, 2001). In *T. brucei*, however, the observed release of cytochrome *c* and the depolarization of the membrane potential are not caused by mitochondrial fission, as they precede mitochondrial fragmentation by 4.5 and 2.5 h, respectively (Figs 2C and 3B). At present, we cannot explain this discrepancy. However, it is interesting to note that the *T. brucei* mitochondrion has 'discoidal cristae' (flat with pinched bases) whereas mammalian mitochondria contain 'flattened cristae'. Discoidal cristae are found in few protozoa and are thought to represent the most ancestral cristae type (Taylor, 1999). The only physiological fission event predicted for the *T. brucei* mitochondrion is expected to occur during cell division and must therefore be tightly regulated temporally and spatially. It is possible that expression of Bax activates this putative mitochondrial fission machinery at the wrong sites and at the wrong time.

Bax-induced effects are reversible

Removing tetracycline from the cell culture stops Bax expression. Interestingly, all three described Bax-induced transitions are, in principle, reversible, as a *T. brucei* population that was expressing Bax for 10 h and therefore consisted of 95% of cells having fragmented mitochondria was able to resume normal growth after a short lag phase (Fig 4A). The lag phase is most likely caused by the fact that outgrowth of the cells requires degradation of the remaining Bax protein. Alternatively, the lag phase might be due to selection of cells that are unable to express Bax. However, we believe this is unlikely as the outgrowing population, on addition of tetracycline, will re-express Bax and respond to it in the same way as the original population (Fig 4A, broken lines). Nevertheless, it is difficult to exclude that the growth of the population is due to selection of the low number of Bax-expressing cells whose mitochondrion never became fragmented. However, Mitotracker staining of the recovering cell culture reveals two populations of mitochondrial membrane-potential-positive cells, one showing the expected single mitochondrion and the other showing unfused mitochondrial vesicles exhibiting a membrane potential (Fig 4B). As the latter are never seen during induction of Bax expression (Fig 2B), these results indicate that at least some cells that have a fragmented mitochondrion are able to re-establish a membrane potential. The detection of these putative intermediates indicates that there is a population of cells in which, after halting of Bax expression, the fragmented mitochondrial vesicles fuse to rebuild the single mitochondrion. The *T. brucei* mitochondrion must therefore have a functional mitochondrial fusion machinery even though fusion is never observed under physiological conditions. Fig 4C shows that cells recovering from 10 h of Bax expression simultaneously restore the membrane potential and the network-like mitochondrial morphology. These results suggest that restoration of the membrane potential precedes mitochondrial fusion, and are in agreement with results from mammalian cells showing that mitochondrial fusion requires an intact membrane potential (Legros *et al*, 2002; Mattenberger *et al*, 2003). Thus, within the first 10 h, all the observed Bax-induced effects are, in principle, reversible, suggesting that neither of the three described events irreversibly damages the mitochondrion. In agreement with this, it was shown that mitochondria of sympathetic neurons after

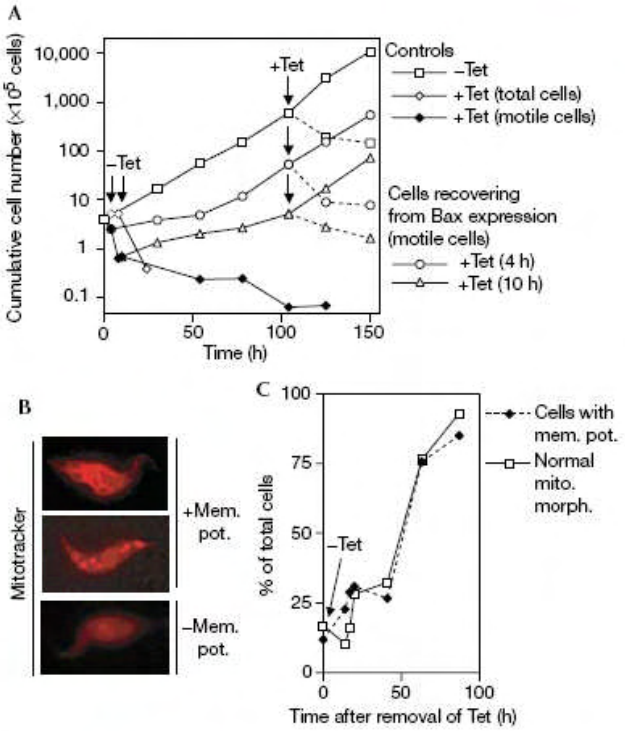

Fig 4 | Recovery from Bax-induced changes. (**A**) Controls: −Tet, growth curves of the *T. brucei* cell line transformed with the human Bax cDNA grown in the absence of tetracycline, all cells are motile; +Tet (total cells) and +Tet (motile cells), same cell line grown in the presence of tetracycline (1 μg/ml) to induce Bax expression, total cell number and motile cells only are indicated, 24 h after induction all cells were immotile. Cell cultures recovering from Bax expression: +Tet (4 h) and +Tet (10 h), growth curves of cell cultures induced for Bax expression for 4 or 10 h, after which expression was stopped by the removal of tetracycline (arrows, −Tet); motile cells only were scored. Broken lines: growth curves of recovering cell cultures in which tetracycline (1 μg/ml) was readded (arrows, +Tet) 95 h after the stopping of Bax expression; motile cells only were scored. (**B**) Three types of Mitotracker staining observed in cultures recovering from Bax expression: top panel, single mitochondrion staining of a completely recovered cell; middle panel, membrane-potential-positive mitochondrial fragments, a staining exclusively observed in recovering populations; lowest panel, membrane-potential-negative cell. (**C**) Time course of the restoration of the membrane potential scored by Mitotracker staining and the normal mitochondrial morphology measured by immunofluorescence using Hsp60 antiserum. The cell culture expressed Bax for 10 h before removal of the tetracycline.

complete release of cytochrome *c* were able to recover fully in the presence of caspase inhibitors (Martinou *et al*, 1999). Prolonged expression of Bax (more than 24 h; Figs 1 and 4A) will kill trypanosomes even in the absence of death effectors. However, the observed Bax-induced killing of *T. brucei* is most likely not of apoptotic nature, but can be explained by the lack of oxidative phosphorylation, a process essential for the survival of procyclic *T. brucei*.

In summary, our work shows that expression of Bax in *T. brucei* causes (i) the release of cytochrome *c* and the loss of oxidative phosphorylation, (ii) the depolarization of the membrane potential and a decline of the intracellular ATP concentration, and (iii) mitochondrial fission. All three events are induced by Bax alone and do not require other apoptotic factors. Furthermore, in contrast to mammalian systems in which the three effects essentially occur simultaneously (Frank *et al*, 2001), they can be temporally separated in *T. brucei* and are, in principle, reversible.

METHODS

Bax-expressing and Bax/Bcl-x_L-coexpressing cell lines. Bax-expressing *T. brucei* cells were prepared as follows: a DNA fragment derived from the cDNA of the wild-type human Bax mRNA was inserted into the trypanosomal expression plasmid pLew100, which carries a tetracycline-inducible procycline promoter (Wirtz & Clayton, 1995; Wirtz *et al*, 1999). The construct was transfected into the insect-stage *T. brucei* strain 29-13, which expresses the tetracycline repressor. Transfection, selection with phleomycine and cloning were performed as described (Beverley & Clayton, 1993). Bax expression was induced by the addition of tetracycline to 1 μg/ml. The Bax/Bcl-x_L-coexpressing cells are based on the Bax-expressing cell line, which was transfected with the Bcl-x_L-expressing plasmid. This plasmid is identical to the Bax-expressing plasmid, except that it contains the human Bcl-x_L cDNA as an insert and a puromycine resistance gene for selection. To remove tetracycline, the cells were reisolated and washed once in media without tetracycline and then resuspended in the same volume of media.

Release of cytochrome *c*. Purification of mitochondria having an intact outer membrane was carried out as described (Hauser *et al*, 1996). Mitochondrial and cytosolic protein extracts from uninduced cells and cells induced for Bax expression for 3 and 5 h, respectively, were resolved on 16% SDS–PAGE, blotted to nitrocellulose and probed with polyclonal rabbit antisera directed against trypanosomal cytochrome *c*. The rabbit antiserum was produced by Eurogentec using the peptides PPKERAALPPGDAVR and QERADL IAYLETLKD as antigens.

ATP production assays. ATP production assays were carried out as described (Allemann & Schneider, 2000; Bochud-Allemann & Schneider, 2002), and a detailed analysis of the different ATP production pathways in *T. brucei* and how they can be distinguished has been published (Bochud-Allemann & Schneider, 2002). Oxidative phosphorylation was measured using either succinate or glycerol-3-phosphate as substrate. Trypanosome-specific mitochondrial substrate level phosphorylation was measured in the presence of antimycine using a combination of pyruvate and succinate as substrates. For all substrates, the reactions were also performed in the presence of atractyloside, a specific inhibitor of the ADP/ATP translocator. Atractyloside blocks the import of ADP and is used to prove that the detected ATP productions are mitochondrial.

To measure the total cellular ATP content, aliquots taken at the indicated times and containing equal cell numbers were treated with 0.01 U/μl of apyrase at 4°C for 5 min and processed in the same way as described above.

Immunofluorescence. Immunfluorescence was performed as described (Sherwin *et al*, 1987). Fixation was carried out using 4% (w/v) of paraformaldehyde in 1 × phosphate-buffered saline (PBS) for 10 min, and cells were permeabilized for 2 min using PBS containing 2% (w/v) of Triton X-100. Expression and localization of Bax were determined using 2 μg/ml of the monoclonal anti-human Bax antibody 2D2 (NeoMarkers, Fremont, CA). To visualize the mitochondrion, a 1:100 dilution of a polyclonal anti-Hsp60 antiserum raised against the isolated yeast protein was used.

Immunoblots. To detect Bax expression on immunoblots, we used a 1:1,000 dilution of the polyclonal rabbit anti-human Bax antiserum (BD Pharmingen) and the SuperSignal West Femto Max Sensitivity Substrate from Pierce for detection.

ACKNOWLEDGEMENTS

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II. RESULTS 2

Ablation of the single dynamin of *T. brucei* blocks mitochondrial fission and endocytosis and leads to a precise cytokinesis arrest.

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Ablation of the single dynamin of *T. brucei* blocks mitochondrial fission and endocytosis and leads to a precise cytokinesis arrest

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Summary

Mitochondrial fission is mediated by dynamin-like proteins (DLPs). *Trypanosoma brucei* contains a single DLP, which is the only member of the dynamin superfamily. We have previously shown that expression of the human proapoptotic Bax in *T. brucei* induces extensive mitochondrial fragmentation. Here we report that Bax-induced mitochondrial fission is abolished in cell lines lacking functional DLP suggesting that the protein is also required for mitochondrial division during the cell cycle. Furthermore, DLP-ablated cells are deficient for endocytosis and as a consequence accumulate enlarged flagellar pockets. Thus, besides its expected role in mitochondrial fission the trypanosomal DLP is required for endocytosis, a function thought to be restricted to classical dynamins. In agreement with its dual function, the DLP localizes to both the mitochondrion and the flagellar pocket, the site where endocytosis occurs. Unexpectedly, ablation of DLP also causes an arrest of cytokinesis. The fact that no multinucleation is observed in the arrested cells argues for a precise cell-cycle block. Furthermore, analysis of a clathrin-knockdown cell line suggests that the cytokinesis arrest is not due to the endocytosis defect. Thus, our results support a working model in which mitochondrial fission triggers a checkpoint for cytokinesis.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/14/2968/DC1

Key words: Trypanosomes, Mitochondria, Endocytosis, Cell cycle

Introduction

Dynamin-like proteins (DLPs) are large modular GTPases consisting of the GTPase, the middle and the GTPase effector domains (Praefcke and McMahon, 2004). Together with the classical dynamins they define the eukaryotic superfamily of the dynamins. Classical dynamins in addition to the three modules found in DLPs also contain a pleckstrin-homology and a proline-rich domain. Whereas DLPs generally function in the division of organelles, such as mitochondria (Otsuga et al., 1998; Smirnova et al., 1998), chloroplasts (Gao et al., 2003; Miyagishima et al., 2003) and peroxisomes (Koch et al., 2003), classical dynamins are required for the scission of a wide range of vesicles including clathrin-coated pits in the secretory pathway (Hinshaw, 2000).

The genome of the parasitic protozoon *Trypanosoma brucei*, unlike most other eukaryotes, encodes only two tandemly linked, 97% identical DLPs (termed TbDLP), which are the sole members of the dynamin superfamily in this organism (Field and Carrington, 2004; Morgan et al., 2004). The two slightly different gene products are probably functionally equivalent, because the few amino acids that differ between them are spread over the entire length of the molecule and represent for the most part conservative substitutions.

T. brucei is ideally suited to study the mitochondrial-linked function of dynamins because it has a single mitochondrion (Tyler et al., 2001). Unlike the very dynamic mitochondria of mammalian cells (Scott et al., 2003), the *T. brucei* mitochondrion does not undergo any fission events throughout the cell cycle. Only before or during cytokinesis does the mitochondrion divide in two in order to allow its transmission to the daughter cells (McKean, 2003). Thus, in *T. brucei* mitochondrial fission must be coordinated with the cell cycle. Furthermore, *T. brucei* is also a good model to study the putative roles TbDLP might play in the secretory pathway. Endocytosis and exocytosis in *T. brucei* are known to be restricted to a small flask-shaped invagination of the plasma membrane containing the base of the flagellum, termed the flagellar pocket (FP) (Field and Carrington, 2004; Overath and Engstler, 2004). Furthermore, all intracellular endosomal compartments are found in a small region in the posterior part of the cell between the FP and the nucleus.

Endocytosis and mitochondrial activity are conversely regulated during the *T. brucei* life cycle. Bloodstream forms in the mammalian host show a ~tenfold higher endocytosis rate than the insect procyclic form (Engstler et al., 2005). Oxidative phosphorylation, on the other hand, is only essential in procyclic forms, which are characterized by having a large network-like single mitochondrion. The mitochondrion of bloodstream forms is much smaller, tube-like and not involved in ATP production (Schneider, 2001). In our work we were

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concentrating on the procyclic form, which has the most active mitochondrion. The aim of our study was to investigate the involvement of TbDLP, the single dynamin of *T. brucei*, in mitochondrial fission and endocytosis. Moreover we wanted to test whether fission of the single *T. brucei* mitochondrion and cell division are independent of each other or whether there is a functional connection between the two events. Practically we approached the problem by inducible RNA interference (RNAi) or by regulated overexpression of a dominant-negative variant of the protein and subsequent analysis of the resulting cellular populations that lack functional TbDLP. The results showed that TbDLP is not only required for mitochondrial fission and endocytosis, but also for completion of cytokinesis.

Results

TbDLP is required for Bax-induced mitochondrial fission

Ablation of TbDLP in procyclic *T. brucei* by RNAi, in agreement with previously published work (Morgan et al., 2004), resulted in a growth arrest within 24 hours. However, unlike previous reports based on Mitotracker-stained cells, the mitochondrial morphology as analyzed by immunofluorescence appeared not to be significantly affected (Fig. 1A). It is known that in apoptotic human cells DLP is recruited by the proapoptotic Bax protein and that this results in extensive mitochondrial fragmentation (Bossy-Wetzel et al., 2003; Karbowski and Youle, 2003). Furthermore, we have previously shown that ectopic expression of human Bax induces mitochondrial fragmentation in *T. brucei* (Crausaz-Esseiva et al., 2004) (Fig. 1B). Thus, to test whether TbDLP is required for the Bax-induced mitochondrial fission, we constructed a cell line in which RNAi-mediated ablation of TbDLP was combined with Bax expression. Interestingly, the growth phenotype of this cell line (Fig. 1C) was essentially identical to the one of the TbDLP-RNAi cell line alone (Fig. 1A). Moreover, the proportion of cells showing mitochondrial fragmentation, which was 90% in the Bax-expressing cell line (Fig. 1B), became reduced to 14% (Fig. 1C). Bax-induced cytochrome *c* release, on the other hand, in agreement with our previous study (Crausaz-Esseiva et al., 2004), was still observed (data not shown). Very similar results were obtained for the cell line expressing the dominant-negative TbDLP (TbDLP-K39A), which is unable to hydrolyze GTP (Yoon et al., 2001) (see supplementary material Fig. S1). In summary, these results show that TbDLP – similarly to its mammalian homologue (Karbowski and Youle, 2003) – is required for Bax-induced mitochondrial fission and strongly suggest that it is also involved in the binary mitochondrial fission observed during cytokinesis.

TbDLP is required for endocytosis

TbDLP is the only member of the dynamin family in *T. brucei* (Morgan et al., 2004). This raises the question whether it might also be required for endocytosis. Indeed, analysis of TbDLP-ablated cells by light and electron microscopy shows an enlargement of the FP (Fig. 2A,B) a phenotype that is very similar to that caused by the ablation of clathrin heavy chain (CLH) (Engstler et al., 2005; Morgan et al., 2004) and actin (Garcia-Salcedo et al., 2004), two proteins known to be required for endocytosis in *T. brucei*. However, in these cases the enlarged FPs were reported to be restricted to the bloodstream stage, whereas in TbDLP-RNAi cells they are

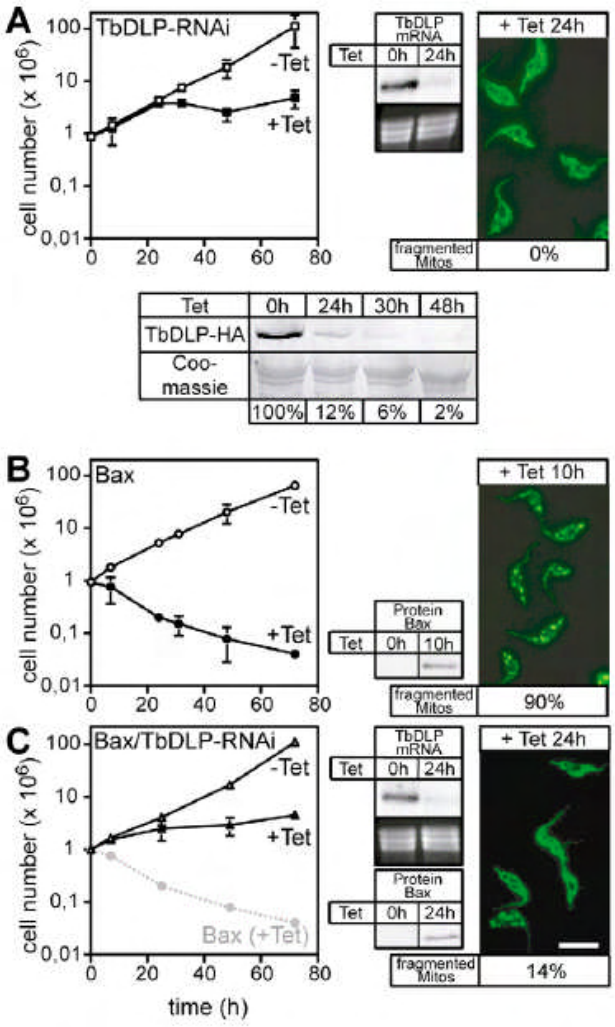

Fig. 1. TbDLP is required for Bax-induced mitochondrial fission. (A) Growth curve of the inducible (–Tet, +Tet) *T. brucei* TbDLP-RNAi cell line. RNAi was confirmed by northern blot (middle panel). Right panel: Immunofluorescence of induced cells using an inner mitochondrial membrane-specific F1-ATPase antiserum. The fraction of cells showing a fragmented mitochondrion is indicated. Bottom panel: Kinetics of TbDLP ablation tested by co-expression of a HA-tagged TbDLP (TbDLP-HA). The tagged protein was detected on immunoblots using anti HA-tag antiserum Y-11 (Santa Cruz Biotechnology). Hours of induction are indicated at the top. The lower half of the panel shows the Coomassie-stained tubulin region and serves as a loading control. (B) Same as A, but data are from a *T. brucei* cell line allowing inducible expression of human Bax (Crausaz-Esseiva et al., 2004). Bax expression was verified by immunoblot. (C) Same as A, but data are from a cell line allowing inducible expression of Bax and RNAi of TbDLP at the same time. The growth curve for the Bax-expressing cell line, as in B, is shown in grey for comparison. Standard errors (n=3-7) are indicated. Bar, 25 μm.

detected in procyclic cells. Concomitant with the appearance of the large FPs in 40-50% of induced TbDLP-RNAi cells, we observe a ~40% reduced endocytosis rate of fluorescently tagged surface proteins (Fig. 2C). Two-channel fluorescence microscopy, which allows the simultaneous scoring of enlarged

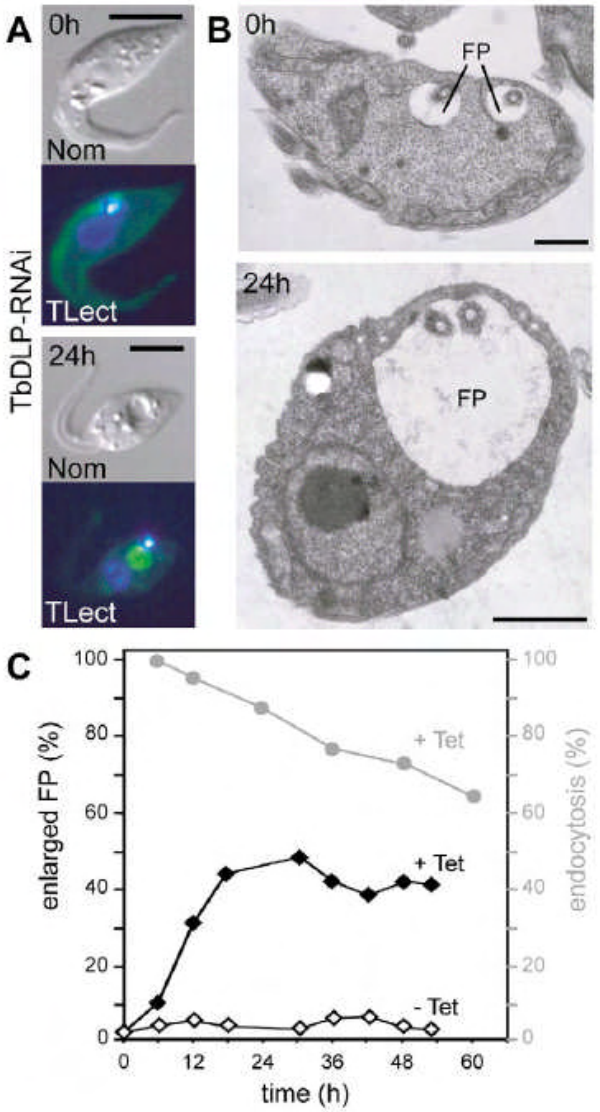

Fig. 2. TbDLP is required for endocytosis. (A) Ablation of TbDLP by RNAi results in enlarged FPs. FP in living cells were visualized by Fluorescein-conjugated tomato lectin (TLect). Nomarski (Nom) images and the merged pictures of the tomato lectin and the DAPI-staining of uninduced (0 h) and induced cells (24 h) are shown. Bars, 5 μm. (B), Analysis of the TbDLP-RNAi cell line by transmission electron microscopy. Bars, 1 μm. (C) Kinetics of appearance of enlarged FPs and loss of endocytic activity during induction of TbDLP-RNAi. FPs were visualized by AMCA sulfo-NHS labeling of surface proteins as described (Engstler et al., 2004). Enlarged FPs in uninduced (–Tet, ◇) and induced (+Tet, ◆) TbDLP-RNAi cells were automatically detected using a series of scripted digital image segmentation steps. Total endocytic activity was measured in the same culture by quantifying the internalized AMCA-labeled surface proteins (+Tet, grey symbols). All values were normalized to the corresponding total cell numbers (n>300 cells) and expressed relative to that of the corresponding uninduced culture.

FPs and endocytosis of a membrane dye within single cells, shows that 92.5±4.3% of cells with an enlarged FP were deficient for endocytosis, whereas the same was true for only 20.8±8.9% of cells having a normal FP. The TbDLP-K39A-expressing cell line exhibited an even stronger phenotype. Within 24 hours, 50% of the cells showed an enlarged FP and a 50% reduced uptake of labeled surface proteins (see supplementary material Fig. S2). Thus, in procyclic *T. brucei* a single DLP is required for both mitochondrial fission and endocytosis.

TbDLP shows a dual localization

To determine where in the cell TbDLP is localized we made use of epitope-tagged versions of the protein containing either an N-terminal or a C-terminal hemagglutinin (HA) tag. The results presented below are for the C-terminally tagged TbDLP. N-terminally tagged TbDLP gave the same results (data not shown). The genes encoding the tagged TbDLP proteins were integrated into their own genomic region in order to achieve expression as close as possible to natural levels (Shen et al., 2001). The resulting cell lines were analyzed by immunofluorescence in combination with confocal microscopy. As expected for a protein that is in part associated with the mitochondrial outer membrane TbDLP is detected in a punctate pattern that is associated but does not colocalize with the mitochondrial matrix marker Hsp60 (Fig. 3A). Furthermore, a strong TbDLP signal is detected next to the mitochondrial DNA. In *T. brucei* the highly concatenated mitochondrial genome, termed the kDNA, is not dispersed throughout the matrix but localized at a precise position opposite to the basal body of the flagellum and thus can easily be visualized by DAPI staining (McKean, 2003; Ogbadoyi et al., 2003). The localization of the kDNA-associated HA-tagged TbDLP was further analyzed by double staining with either the FP marker tomato lectin (Fig. 3B), the antibody YL1/2, which recognizes tyrosinated α-tubulin and serves as a marker for the basal body (Sherwin et al., 1987) (Fig. 3C), or antibodies against the flagellar attachment zone, a structure implicated in the cytokinesis (Kohl et al., 1999) (Fig. 3D). In summary these results show that the TbDLP is peripherally connected to the nucleus-facing side of the FP. It is closely associated but does not overlap with neither the basal body nor the flagellar attachment zone. Thus, in agreement with its dual function in mitochondrial fission and in endocytosis, TbDLP localizes to the mitochondrion (Fig. 3B) and the subregion of the FP where endocytosis is known to occur (Overath and Engstler, 2004) (Fig. 3E).

Lack of TbDLP leads to a precise arrest at the mid-cytokinesis stage

Unlike other eukaryotes, *T. brucei* has a distinct mitochondrial S phase that is initiated immediately before the onset of the longer nuclear S phase (McKean, 2003). Thus, segregation of the replicated kDNAs occurs before the onset of mitosis. The number of nuclei (N) and kDNAs (K) seen in DAPI-stained cells therefore allows us to determine three defined cell-cycle stages showing the 1K1N, 2K1N and 2K2N configurations. Interestingly, analysis of DAPI-stained TbDLP-RNAi cells shows a dramatic accumulation of 2K2N-cells indicative of a cytokinesis block. After 32 hours of induction the 2K2N-cells reach more than 4.5-fold the level (~45%) seen in the uninduced population (Fig. 4A). Most importantly, unlike in many other experiments where inhibition of cytokinesis was observed (Das et al., 1994; Kumar and Wang, 2006; LaCount et al., 2002), very few abnormal cells having configurations

other than 1K1N, 2K1N or 2K2N are detected (Fig. 4A, Others). Of the 2K2N-cells ~75% correspond to a subpopulation (Fig. 4A, NKKN subgroup), where the two kDNAs are localized between the two nuclei, a configuration which we termed NKKN (Fig. 4B, 4C lower panel and 4D). These cells have two opposed motile flagella (Fig. 4C) and resemble cell-cycle 'stage 10' (Sherwin and Gull, 1989) representing a late phase of cytokinesis, corresponding to two daughter cells that lay opposed to each other but which are still connected by their posterior parts. In a wild-type population NKKN cells represent <0.1% indicating that this cell cycle stage is very short. NKKN cells in the induced RNAi cell line, in agreement with the previously reported Mitotracker analysis (Morgan et al., 2004), consistently have a single continuous mitochondrion showing a more collapsed morphology than in uninduced cells (Fig. 4B). Furthermore NKKN cells always have two FPs (Fig. 4C), indicating that ablation of TbDLP does not impair the correct division of the FP. However, as expected from the endocytosis defect, at least one FP is enlarged. Fig. 4D shows that the organization of the flagellar attachment zone, which has been implicated in cytokinesis (Kohl et al., 1999), was not disturbed. Again these results were confirmed by using the TbDLP-K39A cell line (supplementary material Fig. S3).

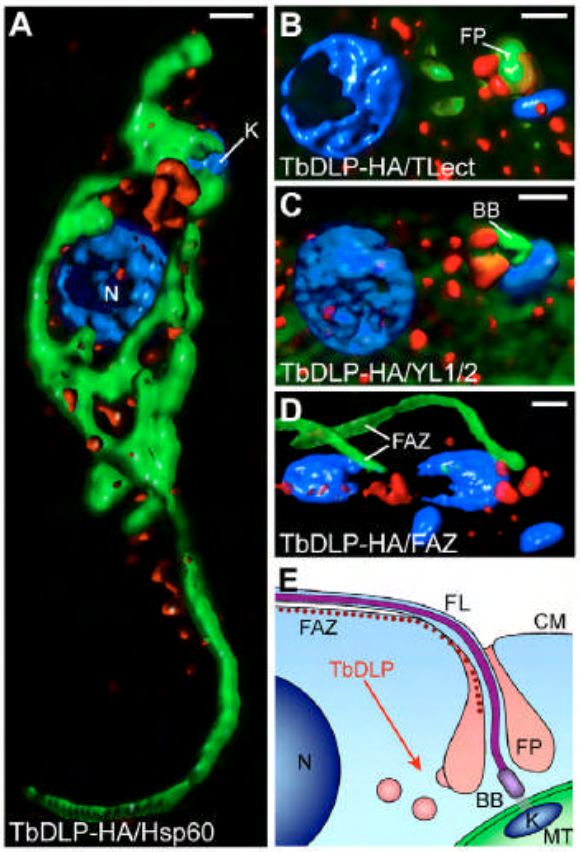

Fig. 3. TbDLP shows a dual localization. A transgenic *T. brucei* cell line expressing C-terminally HA-tagged TbDLP was analyzed by immunofluorescence. HA-tagged TbDLP was detected using anti HA-tag antiserum Y-11 (Santa Cruz Biotechnology) (red). Nuclear (N) and kDNA (K) were visualized by DAPI-staining (blue). The images show 3D-reconstructions from optical sections obtained by confocal microscopy. (A) Co-staining of HA-tagged TbDLP (red) with an antiserum recognizing Hsp60 (green), a marker protein for the mitochondrial matrix. (B) Same as A, but only the nucleus-kDNA region is shown and co-staining was done with the FP marker tomato lectin (TLect) (green). (C) Same as B, but co-staining was done with the antibody YL1/2 (green), which recognizes tyrosinated α-tubulin and serves as a marker for the basal body (BB) (Sherwin et al., 1987). (D) Same as B, but co-staining was done with the monoclonal mouse antibody L3B2 (shown in green), which recognizes the flagellar attachment zone (FAZ) (Kohl et al., 1999). (E) Schematic drawing of the nuclear-kDNA regions of *T. brucei*. The localization of the fraction of TbDLP that is required for endocytosis is indicated relative to intracellular structures and organelles. CM, cell membrane; FL, flagellum; FP, flagellar pocket; MT, mitochondrion. Bars, 1 μm.

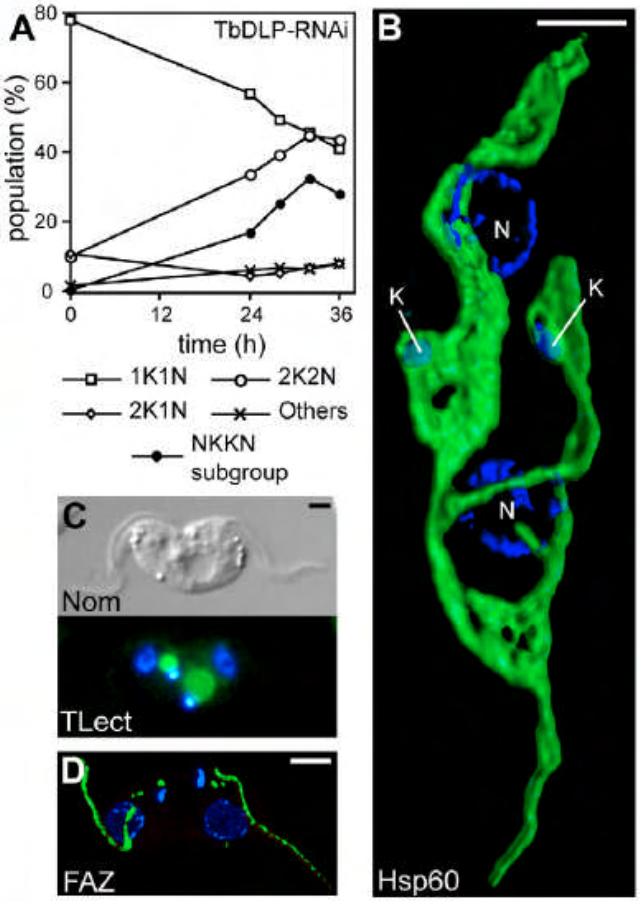

Fig. 4. Lack of TbDLP leads to a specific arrest of cytokinesis. (A) Analysis of nuclei and kDNA configurations of DAPI-stained cells during induction of TbDLP-RNAi. The graph indicates the percentages of cells containing the indicated numbers of nuclei and kDNAs (1K1N, 2K1N, 2K2N and others; n>1000 cells). Percentages of NKKN-cells, a subgroup of 2K2N cells where the two kDNAs are localized between the two nuclei, are also indicated. (B) NKKN-cells have a single mitochondrion. 3D-reconstruction from optical sections obtained by confocal microscopy of an anti-Hsp60 (green) and DAPI co-stained (blue) NKKN cell from the induced TbDLP-RNAi cell line. (C) NKKN cells have enlarged FPs. Visualization of FPs was done as in Fig. 2A. (D) Same as B, but staining was with the antibody L3B2 (green), which recognizes the flagellar attachment zone (FAZ) (Kohl et al., 1999). Bars, 2.5 μm.

Lack of clathrin impairs endocytosis but not cytokinesis

The two key components of the clathrin-mediated endocytosis pathway are dynamin and clathrin. Thus, ablation of clathrin is expected to mimic the endocytosis phenotype observed in the TbDLP-ablated cell lines. In agreement with this, it has been reported that RNAi-mediated depletion of clathrin heavy chain (CLH) in procyclic *T. brucei* causes a growth arrest and a block of endocytosis (Allen et al., 2003; Hung et al., 2004). Exocytosis however was, except for the export of the FP-localized receptor CRAM (Hung et al., 2004), not affected. Thus, in order to test whether endocytosis deficiency can lead to a cytokinesis arrest, we prepared a TbCLH-RNAi cell line. As expected, these cells showed a growth arrest (Fig. 5A) and a reduction of endocytosis (Fig. 5B) after induction of RNAi. However, unlike results described before, concomitant with the growth arrest and in line with the endocytosis defect, we observed that up to 25% of the cells had an enlarged FP (Fig. 5B). Most importantly, even though the enlarged FP and the deficient endocytosis are features also observed in the TbDLP-RNAi cell line, ablation of CLH at no time caused the accumulation of 2K2N cells (Fig. 5C). Thus, the lack of endocytosis or an enlarged FP cannot explain the cytokinesis phenotype caused by the inactivation of TbDLP (Fig. 4A).

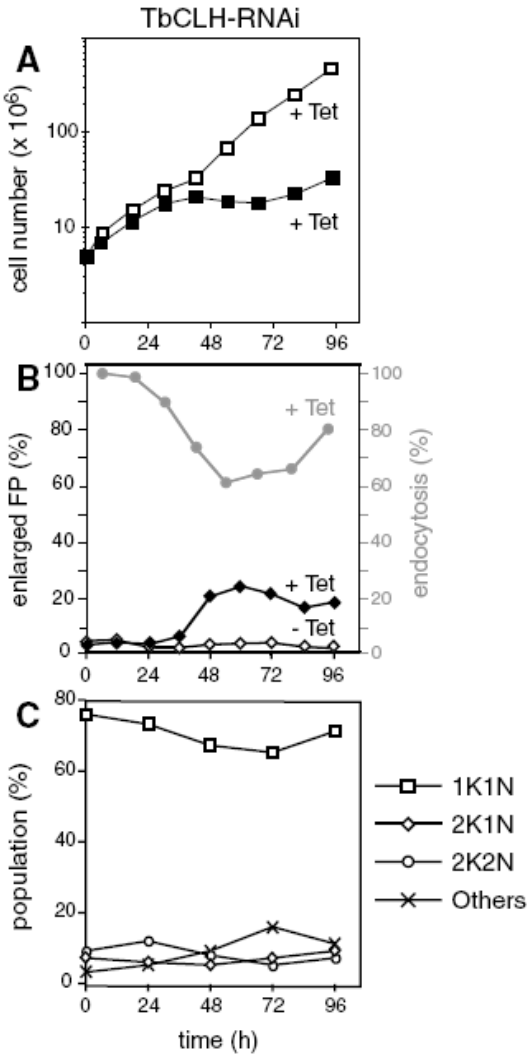

Fig. 5. Lack of TbCLH impairs endocytosis but not cytokinesis. (A) Growth curve of the uninduced and induced TbCLH-RNAi cell line. (B) Kinetics of appearance of enlarged FPs and loss of endocytic activity during induction of TbCLH-RNAi. Analysis was performed as in Fig. 2C. (C) Analysis of nuclei and kDNA configurations of DAPI-stained cells during induction of TbCLH-RNAi carried out as in Fig. 4A.

Discussion

Our results show that TbDLP is required for both mitochondrial fission and – contrary to an earlier report (Morgan et al., 2004) – for endocytosis. At present we cannot explain this discrepancy, but it is interesting to note that the RNAi strategy that was used in our study was based on the expression of a stem-loop RNA whereas in the previous study (Morgan et al., 2004) the two RNA strands were expressed from a single DNA fragment using two opposing T7 promoters. With the stem-loop construct maximal depletion is reached after 1-2 days (Fig. 1A), whereas in the RNAi cell lines produced by Morgan et al. minimal TbDLP levels are reached after 3-4 days. Thus, some of the differences between the results of two studies might be due to the different kinetics and extent of TbDLP depletion. However, it is important to emphasize that whereas the previous study was based on RNAi only, we inactivated TbDLP by either RNAi or expression of the dominant-negative TbDLP-K39A and obtained the same phenotypes. Thus, our results show that clathrin-mediated endocytosis, which generally requires a classical dynamin (Hinshaw, 2000), can be mediated by a DLP suggesting that to some extent classical dynamins and DLPs are functionally interchangeable. Classical dynamins are essentially restricted to metazoans whereas DLPs occur in all eukaryotes. This suggests that DLPs are the most ancestral members of the dynamin superfamily (Elde et al., 2005). *T. brucei* is one of the earliest diverging eukaryotes with a bona fide mitochondrion and its single DLP is required for mitochondrial fission and endocytosis. The unrelated parasitic protozoon *Giardia* branches off the eukaryotic evolutionary tree even earlier, it does not have mitochondria but contains mitochondrial remnant organelles lacking DNA (Tovar et al., 2003), termed mitosomes. Similar to trypanosomes *Giardia* contains a single DLP. Recent experiments have shown that this protein is required for endocytosis, but not for the division of mitosomes (A.H., unpublished results). Thus, these results suggest that the ancestral function of DLPs might have been in endocytosis.

TbDLP-ablated cells are also deficient in cytokinesis. Could it be that TbDLP, besides its role in mitochondrial fission and in endocytosis, has a third function and is directly involved in cell division? We find this unlikely because high-resolution confocal microscopy in dividing 2K2N cells failed to reveal any colocalization of TbDLP with the cleavage furrow (e.g. Fig. 3D and data not shown). Furthermore, it is known that the controls of mitosis and cytokinesis are dissociated in procyclic *T. brucei* and that the mitosis-to-cytokinesis checkpoint, which is operational in mammalian cells, is absent (Das et al., 1994; Kumar and Wang, 2006; LaCount et al., 2002; McKean, 2003; Robinson et al., 1995). Inhibition of cytokinesis therefore invariably causes the appearance of cells having multiple nuclei (Das et al., 1994; LaCount et al., 2002). Inactivation of TbDLP, however, leads to a precise cell-cycle arrest without

appearance of multinucleated cells (Fig. 4A, supplementary material Fig. S3A) providing a further argument against a direct role of TbDLP in cytokinesis.

Analysis of CLH-ablated cells reproduced the same endocytosis defect observed in cells devoid of functional TbDLP but did not cause a cell-cycle phenotype (Fig. 5). This indicates that the cytokinesis phenotype is linked to the defect in mitochondrial fission. A trivial explanation for this would be that the undivided mitochondrion blocks cytokinesis mechanically. We find this unlikely because, in contrast to what would be expected in this case, no central constriction caused by the blocked cleavage furrow is observed in the NKKN cells (Fig. 4C and supplementary material Fig. S3C, upper panel). Moreover, as discussed above, mechanical blockage of cytokinesis would almost certainly cause accumulation of multinucleated cells (Das et al., 1994; LaCount et al., 2002). This is not seen (Fig. 4A and supplementary material Fig. S3A). In fact our results are reminiscent of the block of cytokinesis observed in a bloodstream *T. brucei* cell line ablated for the variable surface glycoprotein (VSG) (Sheader et al., 2005), because multinucleated cells did not accumulate in this case either. It has been suggested, that the observed pre-cytokinesis arrest in this cell line is due to a cell-cycle checkpoint, the function of which would be to coordinate VSG synthesis with cell division (Sheader et al., 2005). Thus, analogous to this interpretation, we propose a model in which inhibition of mitochondrial fission by inactivation of TbDLP prevents completion of cytokinesis by triggering a cell-cycle checkpoint. We think that such a model makes biological sense because the mitochondrial genome of *T. brucei* is of a one-unit nature and because cytokinesis is uncoupled from mitosis (Kumar and Wang, 2006; McKean, 2003; Ploubidou et al., 1999; Robinson et al., 1995). This strongly suggests that novel cell-cycle checkpoints are indeed required. Recent work provides evidence that one of these might be linked to the segregation of the replicated kDNAs (McKean, 2003; Ploubidou et al., 1999). In our model we propose that mitochondrial fission may serve as a checkpoint acting further downstream whose function would be to prevent the production of daughter cells lacking a kDNA or a mitochondrion altogether.

A cell line ablated for Fis1, a protein uniquely involved in mitochondrial fission which acts in concert with DLP (Scott et al., 2003), would in principle be an excellent tool to confirm our results. We did indeed find a putative Fis1 orthologue in the *T. brucei* genomic database. However unfortunately, owing to inefficient RNAi, ablation of the trypanosomal Fis1 was not possible.

Our work shows that in *T. brucei*, TbDLP function links mitochondrial fission, endocytosis and cytokinesis. We believe that the most parsimonious explanation for these results is the existence of a checkpoint for the completion of cytokinesis that monitors mitochondrial fission. However, since alternative explanations cannot entirely be excluded, this must remain a working model at present. Interestingly, some connections of DLPs with cell-cycle progression have been described in other systems. In *Dictyostelium discoideum* a null mutant of an unconventional DLP (DymA) shows pleiotropic defects, one of which concerns the completion of cytokinesis (Wienke et al., 1999). However, unlike in *T. brucei*, the observed cytokinesis arrest was accompanied by the appearance of multinucleated cells and therefore is inconsistent with a specific cell-cycle arrest. RNAi-mediated ablation of the DLP homologue of *Caenorhabditis elegans*, on the other hand, causes embryonic lethality (Labrousse et al., 1999), a result that would be consistent with a mitochondrial segregation defect.

Materials and Methods

Production of transgenic cell lines

TbDLP-RNAi was performed using a stem-loop construct containing the puromycin-resisitant gene as described (Bochud-Allemann and Schneider, 2002). A 502 bp fragment of the TbDLP gene corresponding to nucleotides 1251-1753 was used as an insert. To produce the Bax/TbDLP-RNAi cell line the previously described Bax-expressing cells (Crausaz-Esseiva et al., 2004) selected using phleomycin were transfected with the TbDLP-RNAi plasmid and transfectants were then selected using puromycin and phleomycin. Inducible overexpression of wild-type TbDLP and TbDLP-K39A (where Lys39 was replaced with Ala) was based on the same plasmids used for RNAi. The inducible TbCLH-RNAi cell line was produced by using the construct described by Engstler et al. (Engstler et al., 2005). To produce epitope-tagged variants of TbDLP the recently described one-step PCR-based strategy was used (Shen et al., 2001). Cells were grown in SDM-79 supplemented with 5% (for the 427 strain, used for epitope tagging) or 15% FCS (for 29-13 strain, used for RNAi and overexpression of proteins) and the required antibiotic(s). Transfection, selection with antibiotics, cloning and induction with tetracycline were done as described (McCulloch et al., 2004).

Endocytosis assays

The FPs were analyzed by fluorescence microscopy by labeling with 10 mg/ml Fluorescein-conjugated tomato lectin (Vector Laboratories) (Fig. 2A, Fig. 3B, Fig. 4C and supplementary material Fig. S2A, Fig. S3C). Alternatively, FPs were also visualized by labeling of surface proteins using 1 mM AMCA-sulfo-NHS (Pierce) (Fig. 2C, Fig. 5B and supplementary material Fig. S2B) and incubation for 10 minutes at 0°C followed by 30 minutes at 27°C. Subsequently, after a washing step and fixation of the cells in 4% paraformaldehyde, the FPs were visualized by AMCA fluorescence. It was previously shown that fluorescence of the internalized AMCA-labeled proteins is quantitatively quenched, thus AMCA fluorescence selectively detects labeled proteins on the cell surface and in the FP (Engstler et al., 2004). Enlarged FPs were automatically scored using a series of scripted digital image segmentation steps. This allowed us to determine the fraction of cells at the indicated time points having at least one enlarged FP (Fig. 2C, black curves and supplementary material Fig. S2B, black curves).

To measure endocytosis, surface proteins were labeled with AMCA-sulfo-NHS and incubated as decribed above. Subsequently, AMCA-labeled surface proteins and AMCA-labeled internalized proteins were detected in fixed and permeabilized cells using a rabbit anti-AMCA antibody and an Alexa Fluor 594-conjugated secondary antibody. Cells having internalized AMCA were scored and for each time point the fraction of the total cellular population showing internalized AMCA fluorescence was determined (Fig. 2C, grey curve and supplementary material Fig. S2B, grey curve). Alternatively, endocytosis was measured in living cells by assaying the uptake of the fluorescent membrane dye FM1-43FX (Molecular Probes). AMCA-labeled cells were incubated in the presence of the fluorescent probe for 30 minutes at 27°C, washed with ice-cold PBS and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde. The washing step removes the dye from the plasma membrane and the FP, whereas endocytosed FM1-43FX is retained in endosomal membranes and can be fixed. Two-channel fluorescence microscopy allows the simultaneous scoring for normal or enlarged FPs and internalized FM1-43FX in single cells.

Immunofluorescence and confocal microscopy

Immunofluorescence was performed as described (Sherwin et al., 1987). Fixation was done using 4% (w/v) paraformaldehyde in PBS for 10 minutes and cells were permeabilized for 2 minutes using PBS containing 2% (w/v) Triton X-100. Mitochondrial morphology was assessed by a 1:200 dilution of a polyclonal rabbit anti-F1-ATPase antiserum (gift from D. Speijer, AMC, Amsterdam, The Netherlands) raised against the isolated mitochondrial ATPase of *Crithidia fasciculata* (Fig. 1 and supplementary material Fig. S1) or with 1:1000 dilution of a mouse polyclonal anti-Hsp60 antiserum, raised against recombinantly expressed *T. brucei* Hsp60 fused to gluthathione S-transferase (Fig. 3A, Fig. 4B and supplementary material Fig. S3). Cells were washed with PBS between incubations, and embedded with Vectashield (Vector Labs, Emeryville, CA) supplemented with the DNA-intercalating agent DAPI for detection of nuclear DNA. Fluorescence analysis (Fig. 3, Fig. 4B and supplementary material Fig. S2B) was performed on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using a HCX PL APO (63× /1.3 Glyc Corr) objective and the appropriate laser and photo multiplier settings. Image stacks of 512×512 pixels

were collected using twofold oversampling and further processed using the Leica software, the Huygens Essential deconvolution package (Scientific Volume Imaging, Hilversum, The Netherlands) or Imaris (Bitplane, Zurich, Switzerland). Thresholds for isosurface analysis were calculated automatically and adapted to specific structures manually based on the volume image.

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SUPPLEMENTAL MATERIAL

FIGURE LEGENDS

Supplemental Fig. 1. Expression of the dominant negative TbDLP (TbDLP-K39A) inhibits Bax-induced mitochondrial fission. **(***A***)** Growth curve of a *T. brucei* cell line allowing inducible overexpression of TbDLP. Right panel: Immunofluorescence as in Fig. 1. **(***B***)**, Same as (*A*), but data are for a *T. brucei* cell line allowing inducible expression of TbDLP-K39A. **(***C***)**, Same as (*A*) and (*B*), but data are for a cell line allowing inducible expression of Bax and TbDLP-K39A at the same time. Expression of Bax was verified by immunoblot. The growth curve for the Bax expressing cell line, same as in Fig. 1(*B*), is shown in grey for comparison. Standard errors (n= 3-7) are indicated. Bar = 25 μm.

Supplemental Fig. 2. Expression of the dominant TbDLP-K39A inhibits endocytosis. (*A*) Overexpression of TbDLP-K39A results in enlarged FPs. FP in living cells were visualized by fluorescein-conjugated tomato lectin (TLect). Nomarski (Nom) images and the merged pictures of the tomato lectin and the DAPI-staining of uninduced (0 h) and induced cells (14 h) are shown. Bars = 5 μm. (*B*) Kinetic of appearance of enlarged FPs and loss of endocytic activity after induction of TbDLP-K39A expression. Visualization of the FP in uninduced and induced cells was done by AMCA sulfo-NHS labeling of surface proteins as described (Engstler et al., 2004). Enlarged flagellar pockets in uninduced (-Tet, white diamonds) and induced (+Tet, black diamonds) TbDLP-K39A expressing cells were automatically detected using a series of scripted digital image segmentation steps. Total endocytic activity was measured in the same culture by quantifying the internalized AMCA-labeled surface proteins (+Tet, grey symbols). All values were normalized to the corresponding total cell numbers (n > 300 cells) and expressed relative to the one of the corresponding uninduced cultures. Single cell analysis showed that 89.8±6.2% of cells having an enlarged FP were defective in endocytosis, while 87.4±7.6%of cells with a normal FP showed normal endocytic activity.

Supplemental Fig. 3. Expression of the dominant TbDLP-K39A leads to a specific arrest of cytokinesis. (*A*) Analysis of nuclei and kDNA configurations of DAPI-stained cells during induction of TbDLP-K39A expression. The graph indicates the percentages of cells containing the indicated numbers of nuclei and kDNAs (1K1N, 2K1N, 2K2N and others; n >1000 cells). Percentages of NKKN-cells, a subgroup of 2K2N cells where the two kDNAs

are localized between the two nuclei, are also indicated. (*B*) NKKN-cells have a single mitochondrion. 3D-reconstruction from optical sections obtained by confocal microscopy of an anti-Hsp60 (green) and DAPI co-stained (blue) NKKN-cell from the TbDLP-K39A expressing cell line. (*C*) NKKN-cells have enlarged FPs. Visualization of the FPs was done as in Fig. 2A. Bars = 2.5 μm.

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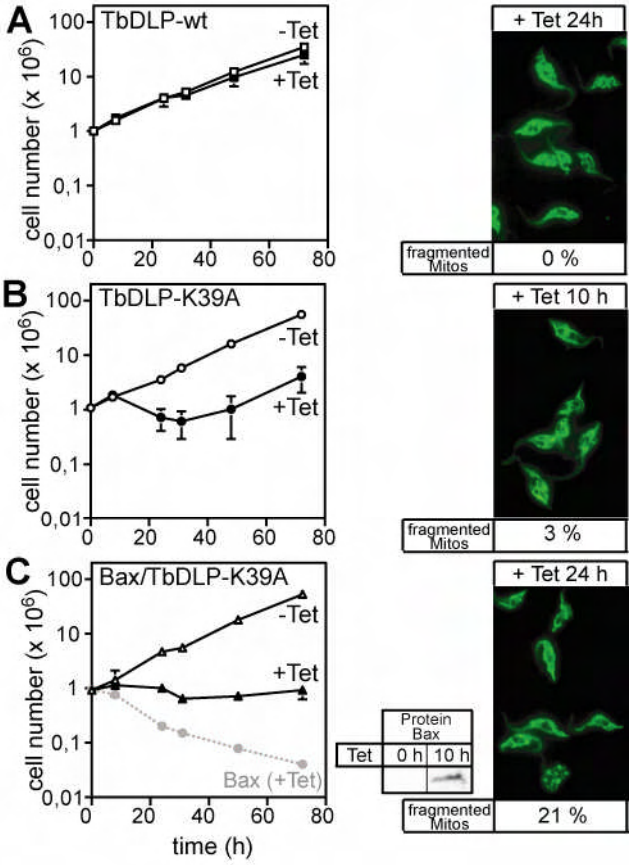
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-Tet
+Tet
cell number (x 10^6)
100
10
1
0,1
0,01
0
20
40
60
80
+ Tet 24h
fragmented Mitos
0 %
B
TbDLP-K39A
-Tet
+Tet
+ Tet 10 h
fragmented Mitos
3 %
C
Bax/TbDLP-K39A
-Tet
+Tet
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time (h)
Protein Bax
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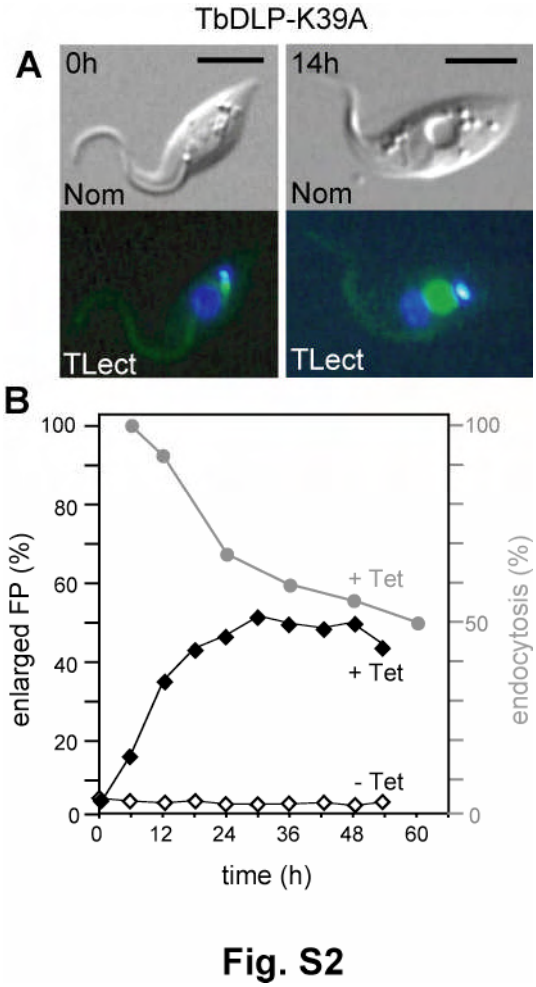
0 h
10 h
+ Tet 24 h
fragmented Mitos
21 %

Fig. S1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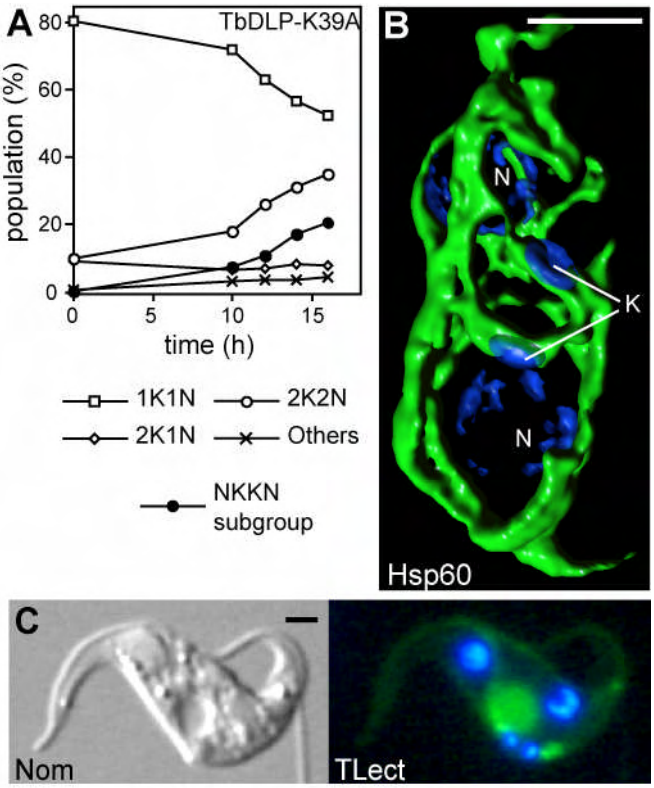
A
0h
14h
Nom
Nom
TLect
TLect
B
100
80
60
40
20
0
enlarged FP (%)
+ Tet
+ Tet
- Tet
100
50
0
endocytosis (%)
0
12
24
36
48
60
time (h)

Fig. S2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TbDLP-K39A
population (%)
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60
40
20
0
0
5
10
15
time (h)
1K1N
2K2N
2K1N
Others
NKKN
subgroup
B
N
K
N
Hsp60
C
Nom
TLect

Fig. S3

The putative Fis1 homologue of *Trypanosoma brucei*: Preliminary results

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Abstract

Two proteins of the mitochondrial fission machinery have been conserved throughout evolution: the dynamin-related protein (termed Drp1, Dnm1 or DLP depending on the species) and Fis1, an integral membrane protein of the outer mitochondrial membrane. We have previously characterized the DLP of the parasitic protozoon *Trypanosoma brucei* (11). In this study we identified the Fis1 homologue, termed TbFis1, of *T. brucei*. However, we were not able to determine the intracellular localization of the protein. Furthermore over-expression of TbFis1 as well as disruption of both alleles of the TbFis1 gene did not give an obvious phenotype, neither on growth nor on mitochondrial morphology. These results suggest that the function of Fis1, and more generally the mechanism of regulation of the mitochondrial fission, may differ in *Trypanosoma brucei* when compared to other eukaryotes.

Introduction

Mitochondria play important roles in the cellular metabolism, ATP production and apoptosis (1). They are dynamic organelles, undergoing frequent fission and fusion events. The number and shape of mitochondria are maintained by an equilibrium between fission and fusion reactions. Thus, changes in the balance of these two processes lead either to excessive fusion, which forms net-like mitochondria, or to excessive fission resulting in a large number of small mitochondria (1, 2). Such morphological variations are important to adapt mitochondrial functions to the needs of the cell. Moreover, since mitochondria cannot be formed *de novo*, fission is essential to form new organelles that can be distributed to the two daughter cells (1).

In yeast, the mitochondrial fission machinery consists of at least three different proteins. The main factor is Dnm1 (known as Drp1 in mammals), a large GTPase belonging to the subfamily of the dynamin-like proteins (2-4). The dynamin superfamily includes both classical dynamins, that are for example required for membrane scission of clathrin-coated vesicles during endocytosis, and the dynamin-like proteins, involved in division of organelles such as mitochondria and chloroplasts (5). Dnm1, responsible for mitochondrial fission, is mainly cytosolic but can translocate to fission sites of the outer mitochondrial membrane (2, 4). The other known components of the mitochondrial fission machinery mediate the targeting of Dnm1 to the precise sites where fission has to occur (1, 2). The most important of these appears to be Fis1 (2, 6). Fis1 forms a complex with Mdv1, a WD40 protein that acts as an adaptator which binds both Fis1 and Dnm1 (7). Interestingly, only Dnm1 and Fis1 have homologues in other organisms, whereas Mdv1 seems to be restricted to yeast (1).

The parasitic protozoon *Trypanosoma brucei* has a single mitochondrion only (9), and thus is ideally suited to study the mechanisms of mitochondrial fission. Moreover, contrary to other organisms, the mitochondrion of *T. brucei* does not undergo any fission or fusion events throughout the cell cycle. The only fission event occurs prior to or during cytokinesis to allow transmission of one complete mitochondrion to each daughter cell (10). The homologue of Dnm1, termed TbDLP, has been already studied in *T. brucei* (11, 12). Interestingly, this protein was not only shown to be required for mitochondrial fission, but is also involved in endocytosis, a function generally attributed to classical dynamins, that are absent in *T. brucei*. Furthermore, it was shown that TbDLP-dependent mitochondrial fission is required for

completion of cytokinesis, suggesting that mitochondrial fission might be a checkpoint for cell division (11).

In the present work, we identified the putative Fis1 homologue in *Trypanosoma brucei*. Interestingly gene knock-out studies showed that TbFis1 may act in mitochondrial fission in a quite different way than its homologues in other organisms.

Material and Methods

Cells

Procyclic *T. brucei* cells were cultured at 27°C in SDM-79 supplemented with 5% or 15% of FCS, for the wild-type 427 and the inducible 29-13 strains respectively (13), and the required antibiotics. The medium lacking glucose (SDM-80) was prepared as described (14), and supplemented with 15% dialyzed FCS and the required antibiotics.

Production of transgenic cell lines

To localize TbFis1, we replaced one TbFis1 allele in the wild-type 427 strain using the recently described one-step PCR (15). A PCR fragment containing the genes for resistance to blasticidin, the intergenic region, a promoter and one copy of the hemagglutinin tag was integrated at the 5'-end of the TbFis1 using 70bp-long primers. Epitope tagging was also performed using a 10 amino acids (EVHTNQDPLD) epitope of the structural yeast protein Ty1, which is recognized by the monoclonal antibody BB2 (16). Sequences of the full-length TbFis1 only (for over-expression) or including this Ty1 tag at the N-terminus of the gene (for localization) were cloned in a derivative of pLew100 containing the puromycin resistance gene in order to allow tetracycline inducible expression (13).

RNAi of TbFis1 was done in a stem-loop plasmid containing the puromycin resistance gene (17). A 473bp fragment corresponding to nucleotides 169 to 641 was used as insert. For gene knock-out, 500bp of the 5' UTR and 280bp of the 3'UTR TbFis1 gene were inserted each at one end of a resistance gene (phleomycin or puromycin) in a derivative of pBluescript II KS+. Linearization was done using *Xho*I and *Bam*HI endogenous restrictions sites.

To produce the Bax/TbFis1-KO strain, we transfected TbFis1-KO cells with the previously described plasmid for expression of the human Bax protein (18), where phleomycin resistance was changed in blasticidin resistance.

Transfection, selection with antibiotics, cloning and induction with tetracycline were done as described (19).

Immunofluorescence

Immunofluorescence was done as described (20). Cells were fixed for 10 minutes with 4% (w/v) paraformaldehyde in PBS 1x and permeabilized for 2 minutes with 2% (w/v) NP-40 in PBS 1x. Mitochondria were stained using anti-F1-ATPase antiserum (gift from D. Speijer, AMC, Amsterdam, The Netherlands) raised against the isolated mitochondrial ATPase of

Crithidia fasciculate (dilution 1:200). Cells were washed with PBS 1x between incubations and embedded with Vectashield (Vector Labs, Emeryville, CA) supplemented with the DNA-intercalating agent DAPI for detection of DNA.

Southern blot

Genomic DNA was extracted as described previously (21). Five micrograms of DNA were digested with *Hinc*II and separated on 0.8% agarose gel. Southern Blot analysis was performed using standard procedure (22). Detection was done using radiolabeled fragment of the 5'UTR of TbFis1 (500bp).

Results and Discussion

Identification of Fis1 homologue in Trypanosoma brucei

To identify the Fis1 homologue of *T. brucei*, we performed a BLAST analysis against the trypanosome genomic database of the Wellcome Trust Sanger Institute using the human hFis1 as template. This search identified the ORF Tb10.6k15.1880 as putative Fis1 homologue. This ORF of 726bp encodes a 241 amino acids polypeptide with a predicted molecular mass of 26.8kDa. The protein shows an overall identity of ~14% and a homology of ~41% with the human hFis1. The highest similarity was found in the C-terminal part of the protein (~24% identity and ~73% homology). This part of the protein shows structural features common to all Fis1 homologues such as tetratrico-peptide repeat (TPR) motif fold (23, 24) and a single transmembrane domain at the extreme C-terminal end of the protein. Whereas the human and yeast Fis1 proteins contain two TPR domains each, the trypanosomal protein encodes only a single one. The TPR domains of the yeast and the human Fis1 form a concave hydrophobic surface which allows binding to other proteins including Dnm1 homologue (23). Furthermore, the transmembrane domain was shown to be essential for the mitochondrial localization of Fis1 (25).

In comparison to the other Fis1 homologues, the trypanosomal protein contains two insertions: A small N-terminal one and an about 70 amino acids long in the central part of the protein. These insertions explain the low degree of similarity with Fis1 homologues of other specise (Fig.1). However screening different genomes with the trypanosomal protein generally results in best matches to Fis1 homologues. Based on this we concluded that the gene Tb10.6k15.1880 indeed encodes the trypanosomal Fis1 protein.

Attempts to localize TbFis1

The localization of the yeast and human proteins revealed that Fis1 is evenly distributed on the outer mitochondrial membrane (6, 25), with the N-terminus exposed to cytoplasm (25). Thus we wanted to determine whether TbFis1 shows the same intracellular localization. However neither N-terminal addition of a hemagglutinin tag in the correct genome context of TbFis1 nor ectopic over-expression of the full-length protein containing a N-terminal Ty1 tag resulted in a detectable signal by immunofluorescence or western blots (not shown).

A possible explanation for the absence of a detectable signal when the tagged TbFis1 is expressed under the control of the endogenous promoter could be that only very little protein is present in the cell or that the protein might only be expressed at a specific point during the

cell cycle (10). This idea is consistent with the fact that only a weak signal is detected for TbDLP even though this protein is known to form homo-oligomers to promote fission (4, 11, 26), whereas Fis1 does not interact with itself (1, 2), and thus is less concentrated than TbDLP. Furthermore, also in human cells, the endogenous hFis1 was not detectable by antibody it was suggested that it is a consequence of the low level of protein in the cell (27). However, this cannot explain the absence of signal when the epitope tagged TbFis1 is over-expressed. But it could be in this case that the over-expression affects the function, the localization or the stability of the protein (15).

Over-expression of TbFis1 does not affect mitochondrial morphology

Dnm1/Drp1-targeting on mitochondria depends on Fis1 (6, 27). Thus an important function of Fis1 consists in limiting mitochondrial fission. Indeed over-expression of hFis1 in mammalian cells results in excessive mitochondrial fission leading ultimately to apoptosis (25). In yeast on the other hand, Fis1 over-expression promotes mitochondrial fusion. An explanation for these contradictory results could be that in yeast Fis1 has a secondary function and limits fission by blocking an irreversible step mediated by Dnm1 (28). Thus, even if the exact mechanisms to control fission are not identical, the mammalian and yeast Fis1 homologues both appear to play important roles in limiting the Dnm1/Drp1-dependent mitochondrial fission.

Thus, we expected to see a mitochondrial phenotype when TbFis1 is over-expressed in *T. brucei*. However we did not observed any significant change in growth, mitochondrial morphology or cell cycle progression (Fig. 2). Thus, the role if any TbFis1 plays in mitochondrial fission in *T. brucei* remains to be investigated.

TbFis1 is not essential for growth

Fis1 disruption in both yeast and mammalian cells results in extensive mitochondrial fusion leading to decrease in the number of mitochondria and a concomitant increase in the size of the organelles (6, 25, 27). In *T. brucei*, we expected that the absence of Fis1 would mimic the mitochondrial phenotypes observed when TbDLP is depleted, meaning growth arrest, inhibition of mitochondrial fission and accumulation of cells in the last cell cycle stage before completion of cytokinesis (11). Inhibition of gene function by RNA interference (RNAi) has been well developed in *T. brucei* (29). However in the case of TbFis1 this method was not successful (data not shown).

As an alternative to RNAi we decided to knock-out the gene coding for TbFis1. In order to do so, we consecutively replaced the two alleles of endogenous TbFis1 by the two resistance genes phleomycin and puromycin respectively. The insertions of the resistance genes into both alleles were monitored by Southern blots (Fig. 3A). Since we were able to obtain a strain that lacks both TbFis1 alleles (TbFis1-KO), we concluded that TbFis1 is not essential for *T. brucei* normal growth. Moreover, depletion of TbFis1 seems also not to affect the morphology of the mitochondrion (not shown). This is surprising, since in all other organisms depletion of Fis1 strongly affects mitochondrial morphology (6, 27, 30).

ATP production in the procyclic *T. brucei* depends mainly of two mitochondrial pathways: oxidative phosphorylation and substrate level phosphorylation. This last one occurs in the ASCT cycle, and depends on glucose (17). Thus when glucose is missing, ATP can only be produced through oxidative phosphorylation, which requires components that are mainly encoded on the mitochondrial genome. Therefore, if *T. brucei* cells grow in a medium without glucose, their survival depends to 100% on efficient oxidative phosphorylation (14). However, when cells lacking TbFis1 were tested on this medium, they grew as well as wild type cells, suggesting that Fis1 is not required to maintain oxidative phosphorylation (Fig. 3B).

TbFis1 is not involved in Bax-induced mitochondrial fragmentation

Mitochondrial fission proteins are known to play important roles in the mammalian cell death pathway (31, 32). During apoptosis, mitochondria are invariably fragmented in a Dnm1/Drp1-dependent manner (31). Fis1 has been shown to play an essential role in the regulation of apoptosis, acting upstream of Drp1/Dnm1. Thus, hFis1 unlike Drp1 is required for Bax translocation to mitochondria (32). However, its function seems to be different in mammals and yeast: Fis1 depletion strongly inhibits apoptosis in mammalian cells (32), whereas disruption of its homologue in yeast enhances programmed cell death and promotes mitochondrial fusion (28).

In *T. brucei,* we have shown that ectopic expression of the human Bax protein induces mitochondrial fragmentation and consecutive cell death (18), and that TbDLP is required for this process (11). Thus to know whether TbFis1 is also implicated in these Bax-induced events, we expressed the human Bax protein in TbFis1-KO cells and monitored growth and mitochondrial fragmentation after induction of Bax expression. Surprisingly we observed that

Bax-induced cell death is maintained even when no TbFis1 is present in the cells (Fig. 3C, 3D).

Function of TbFis1 in Trypanosoma brucei

The results presented here are not consistent with the function of Fis1 described in other organisms. Indeed, neither over-expression nor removal of TbFis1 affects mitochondrial morphology in *T. brucei*. This is different to all other organisms where Fis1 homologues were studied (6, 25, 27, 30). Moreover, disruption of the TbFis1 gene did not result in the same phenotypes that are observed in cells lacking TbDLP (11), even though two proteins are known to interact (either directly or indirectly) and act in the same pathway (7, 27).

The protozoon *T. brucei* is one of the earliest diverging eukaryotes with a bona fide mitochondrion. Thus the mechanism of regulation of mitochondrial fission could be different from the one in higher organisms. Interestingly, none of the conserved components of the mitochondrial fusion machinery has been found in the genome of *T. brucei* (not shown). Therefore, the assumption that mitochondrial morphology is the result of balanced fission and fusion events might not be valid in *T. brucei* and the regulation of mitochondrial morphology in trypanosomes might be determined in a different way.

In higher organisms, Dnm1/Drp1 acts strictly in mitochondrial fission. The protein is mainly localized in the cytosol and is targeted to mitochondria in a Fis1-dependent manner (1, 2). However, the Dnm1 homologue in *T. brucei*, TbDLP, is also involved in endocytosis (11). Thus, due to its two specific functions and consequently two different intracellular localizations, the mechanism of targeting of TbDLP must be more complex than in other organisms. One possibility would be that TbDLP itself contains a mitochondrial targeting sequence, and thus that Fis1 plays only a minor role in the recruitment of TbDLP to mitochondria.

Furthermore also the structure of the trypanosomal TbFis1 shows some differences to its counterparts in other organisms. Fis1 proteins normally contain two TPR motifs forming a concave hydrophobic surface required for interactions with other proteins (23, 24). However TbFis1 has only a single TPR domain and has a long additional insert in the middle part of the protein (Fig. 1). Thus it is possible that these two features could alter the function of TbFis1.

On the other hand, it could also be that TbFis1 is not the true homologue of Fis1. In this case the function of Fis1 may have been taken over by a completely different unknown protein.

Thus we can conclude that *Trypanosoma brucei* is an excellent model to study the regulation of mitochondrial division, showing some interesting variations when compared with other eukaryotes.

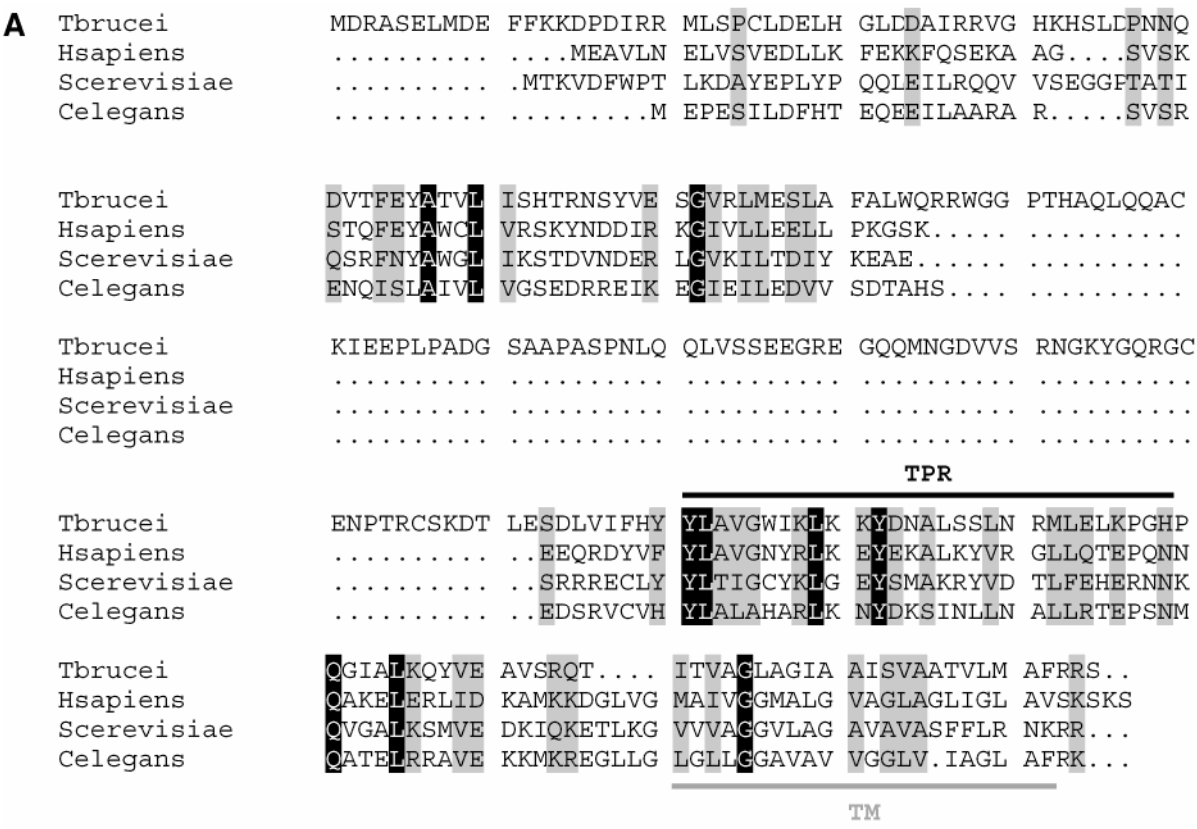
Figure legends

Fig. 1: Identification of the Fis1 homologue in *T. brucei.* A) Amino acids alignment of Fis1 homologues of *T. brucei*, *H. sapiens*, *S. cerevisiae* and *C. elegans.* The sequences were aligned using the CLUSTALW program with default parameters. Strictly conserved residues and conservative replacements are shown in black and grey boxes, respectively. TPR-like and transmembrane domains of TbFis1 are underlined in black and grey, respectively. B) Phylogenic tree based on a multiple alignment of Fis1 homologues of *T. brucei, A. thaliana, S. cerevisiae, C. elegans, M. musculus, H. sapiens* and *B. taurus*. The tree was constructed by using the program TREEVIEW, which is available on:
http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

Fig. 2: Over-expression of TbFis1 shows no effect on growth, mitochondrial morphology and cell cycle progression. A) Growth curve in the presence and the absence of tetracycline of a strain over-expressing TbFis1. B) Immunofluorescence of cells over-expressing TbFis1 before (0h) and after 20h of induction, using an inner mitochondrial membrane-specific F1 ATPase antiserum (green), merged with DAPI staining (blue). Upper panel: Nomarski picture of the stained cells. C) Analysis of kDNA and Nuclei configurations of DAPI-stained cells before and after 20h of induction of TbFis1 over-expression. The graph indicates percentages of cells containing the indicated numbers of kDNAs and nuclei (1K1N, 2K1N and 2K2N). n > 150 cells.

Fig. 3: TbFis1 is not essential for normal growth or maintenance of mitochondrial morphology. A) Depletion of the two alleles of TbFis1 in TbFis1 Knock-out (TbFis1-KO) cell line was confirmed by Southern Blot. Isolated DNAs of 29-13, TbFis1-KO 1st allele and TbFis1-KO 2nd allele strains were digested with *Hinc*II and recognized by the 5'UTR of TbFis1. Digestion of wild-type allele results in 4000bp fragment, whereas replacement of TbFis1 by phleomycin/puromycin resistance genes results respectively in 3200/3300bp fragments. The upper band in 2nd allele KO corresponds probably to undigested DNA. 2-Log was used as DNA marker. B) Growth curve of TbFis1-KO strain in presence or in absence of glucose (red curve). Growth of 29-13 cell line was used as control (black curve). C) Growth curves in presence and in absence of tetracycline of the strains allowing inducible expression of human Bax in 29-13 (black curve) (18), and in TbFis1-KO background (red curve). D) Mitochondrial fragmentation in cells expressing Bax in 29-13 and in TbFis1-KO background.

The graph indicates the percentage of cells with fragmented mitochondria in uninduced cells and after 8h of induction. n = 150-250.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Hsapiens ....MEAVLN ELVSVEDLLK FEKKFQSEKA AG....SVSK
Scerevisiae ..... .MTKVDFWPT LKDAYEPLYP QQLEILRQQV VSEGGPTATI
Celegans ....M EPESILDFHT EQEEILAARA R.....SVSR
Tbrucei DVTFEYATVL ISHTRNSYVE SGVRLMESLA FALWQRRWGG PTHAQLQQAC
Hsapiens STQFEYAWCL VRSKYNDDIR KGIVLLEELL PKGSK..... .....
Scerevisiae QSRFNYAWGL IKSTDVNDER LGVKILTDIY KEAE..... .....
Celegans ENQISLAIVL VGSEDRREIK EGIEILEDVV SDTAHS.... .....
Tbrucei KIEEPLPADG SAAPASPNLQ QLVSSEEGRE GQQMNGDVVS RNGKYGQRGC
Hsapiens ....
Scerevisiae ....
Celegans ....
TPR
Tbrucei ENPTRCSKDT LESDLVIFHY YLAVGWIKLK KYDNALSSLN RMLELKPGHP
Hsapiens ....EEQRDYVF YLAVGNYRLK EYEKALKYVR GLLQTEPQNN
Scerevisiae ....SRRRECLY YLTIGCYKLG EYSMAKRYVD TLFEHERNNK
Celegans ....EDSRVCVH YLALAHARLK NYDKSINLLN ALLRTEPSNM
Tbrucei QGIALKQYVE AVSRQT.... ITVAGLAGIA AISVAATVLM AFRRS..
Hsapiens QAKELERLID KAMKKDGLVG MAIVGGMALG VAGLAGLIGL AVSKSKS
Scerevisiae QVGALKSMVE DKIQKETLKG VVVAGGVLAG AVAVASFFLR NKRR...
Celegans QATELRRAVE KKMKREGLLG LGLLGGAVAV VGGLV.IAGL AFRK...
TM
B
Athaliana
Tbrucei
Scerevisiae
Celegans
Mmusculus
Hsapiens
Btaurus

Fig. 1

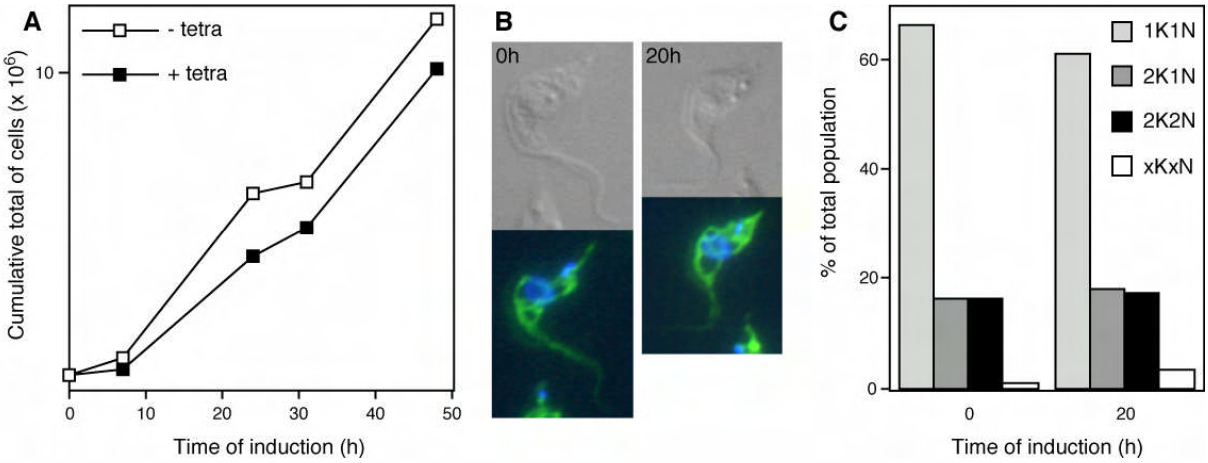
A
- tetra
+ tetra
Cumulative total of cells (x 10^6)
10
0
10
20
30
40
50
Time of induction (h)
B
0h
20h
C
1K1N
2K1N
2K2N
xKxN
% of total population
60
40
20
0
0
20
Time of induction (h)

Fig. 2

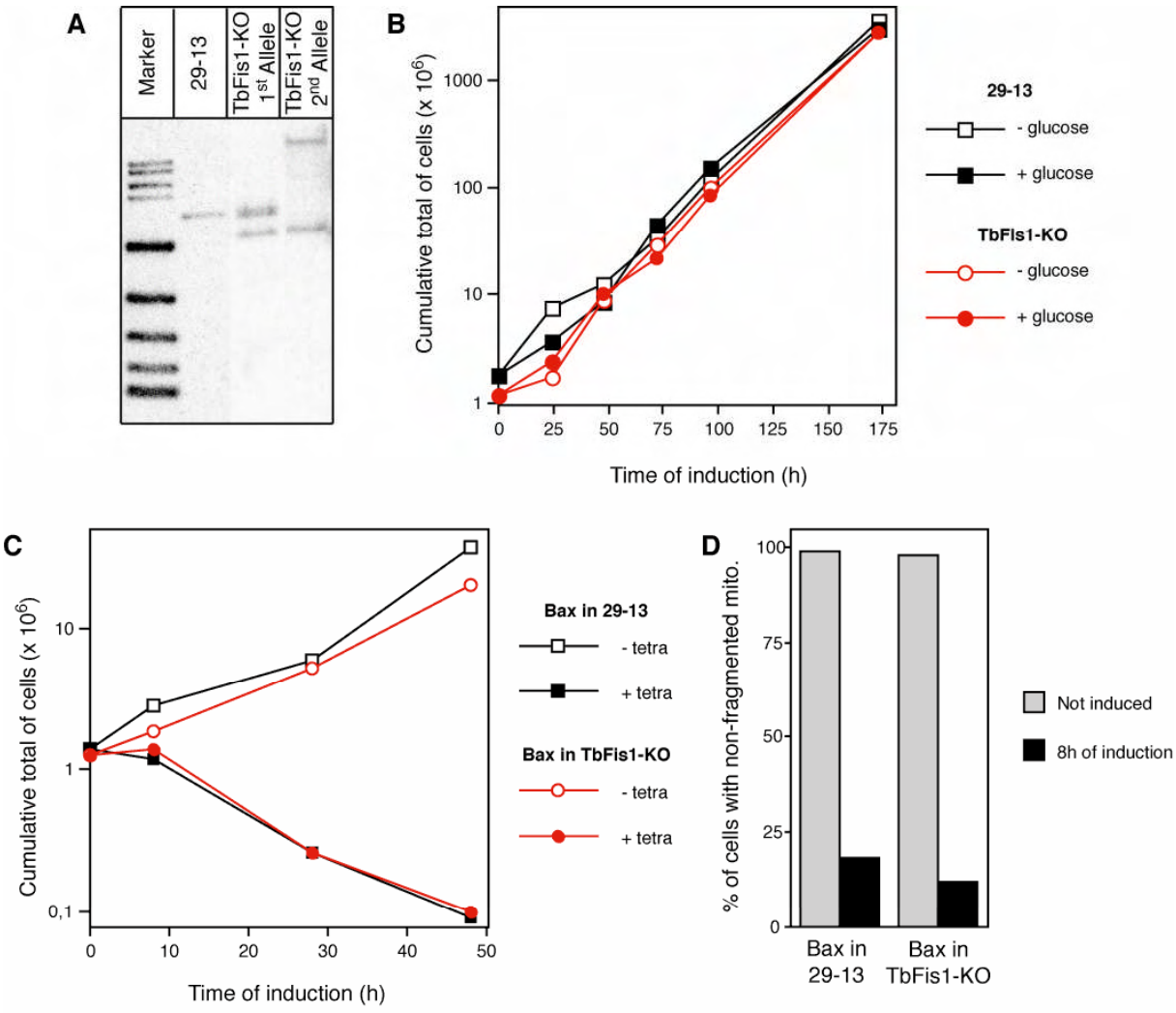
A
Marker
29-13
TbFis1-KO 1st Allele
TbFis1-KO 2nd Allele
B
Cumulative total of cells (x 10^6)
1000
100
10
1
0
25
50
75
100
125
150
175
Time of induction (h)
29-13
- glucose
+ glucose
TbFis1-KO
- glucose
+ glucose
C
Cumulative total of cells (x 10^6)
10
1
0,1
0
10
20
30
40
50
Time of induction (h)
Bax in 29-13
- tetra
+ tetra
Bax in TbFis1-KO
- tetra
+ tetra
D
% of cells with non-fragmented mito.
100
75
50
25
0
Bax in 29-13
Bax in TbFis1-KO
Not induced
8h of induction

Fig. 3

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II. Results 4

Depletion of an outer mitochondrial membrane protein leads to misplacement of the kinetoplast DNA in *Trypanosoma brucei*

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Abstract

The mitochondrial genome of the parasitic protozoon *Trypanosoma brucei* is found in a discrete structure termed the kinetoplast (or kDNA). Because of its one unit characteristic, kDNA duplication and segregation is highly regulated. During the cell cycle of *T. brucei*, the position of the kDNA is dynamic. Furthermore the kDNA also occupies distinct positions in the cell depending on the life cycle stage of *T. brucei*. All these changes of the kDNA position are known to be microtubule-mediated. In this study we show that TbMiX, a trypanosomatid-specific protein that is evenly distributed on the outer mitochondrial membrane, is required for the correct positioning of the kDNA in procyclic *T. brucei.* Moreover, we show that misplacement of the kDNA does not affect cytokinesis. Finally, we present evidence that TbMiX links the mitochondrion to the subpellicular microtubule cytoskeleton, suggesting that the cytoskeleton plays an important role in determining the position of the kDNA.

Introduction

The parasitic protozoon *Trypanosoma brucei* is responsible for transmission of the Nagana disease in cattle and human sleeping sickness in Africa. This devastating disease affects >0.5 million people of which 70'000 die each year (1). *T. brucei* is transmitted between mammalian hosts by *Glossina*, known as the tsetse fly. During its life cycle, the parasite shows different morphological forms. In the bloodstream of the mammalian hosts, they are elongated and actively proliferate. This stage is referred to as the long slender form. Then cell density increases and the long slender form differentiates into the non-dividing stumpy form. When transmitted to the fly, trypanosomes initially establish a midgut infection and differentiate to the proliferative procyclic form. Finally they migrate to the salivary glands where they transform to the epimastigote and finally to the metacyclic form, in preparation for their transmission to mammalian host (1, 2).

T. brucei is one of the earliest diverging eukaryotes, which has bona fide mitochondria (3). In contrast to most other eukaryotes it has a single mitochondrion only, which undergoes extensive morphological and biochemical changes during the trypanosomal life cycle (1). A unique feature of the trypanosomal mitochondrion is the organization of its genome. Generally mitochondrial genomes are distributed all over the matrix. In trypanosomes however it is restricted to a discrete structure termed the kinetoplast (or kDNA) (1, 3). The kDNA is linked to the basal body of the flagellum across the mitochondrial membranes by a tripartite attachment complex (4). Thus its replication is closely linked to basal body duplication and segregation (4-6). The position of the kDNA also depends on the life cycle stage. Thus, the mitochondrial genome is found at the very posterior end of the cell in bloodstream forms, whereas in procyclic cells it lies between the nucleus and the posterior end. Finally, in epimastigote forms, the kDNA is anterior to the centrally localized nucleus (1, 2). All the changes of kDNA positions that occur during differentiations are microtubule-mediated (2). Thus the cytoskeleton of *T. brucei* composed of a microtubule corset that underlies the cell membrane is not only required for the maintenance of the cell shape but is also implicated in organelle positioning and segregation (1).

In this study we have characterized the *T. brucei* homologue of MiX (for Mitochondrial protein X), a protein restricted to kinetoplastidae that was initially studied in *L. major*. Heterozygous deletion of the MiX gene in *L. major* showed pleiotropic effects including alterations in cell morphology, mitochondrial disorganization and a reduction of virulence (Uboldi et al, submitted). Analysis of MiX function in procyclic *T. brucei* showed that the protein is localized on the outer mitochondrial membrane and is required for the positioning of the kDNA. Furthermore, we provide evidence that the protein links the mitochondrion to the cytoskeleton of *T. brucei.*

Material and Methods

Cells

Procyclic *T. brucei* cells were cultured at 27°C in SDM-79 supplemented with 5% or 15% of FCS, respectively for the wild-type 427 and the inducible 29-13 strains (7) and the required antibiotics.

Production of transgenic lines

A 10 amino acids (EVHTNQDPLD) epitope of the major structural protein of yeast Ty1, which is recognized by the monoclonal antibody BB2 was used as a tag to determine the localization of TbMiX (8). The sequence of the full-length TbMiX including the C-terminal Ty1 tag was cloned into a derivative of pLew100 containing the puromycin resistance gene. This plasmid allows tetracycline inducible expression of the tagged protein (7).

RNAi of TbMiX was done using a stem-loop plasmid containing the puromycin resistance gene (9). The first 349bp of the TbMiX ORF were used as insert.

Transfection, selection with antibiotics, cloning and induction with tetracycline were done as described (10).

Immunofluorescence

Immunofluorescence was done as described (11). Cells were fixed for 10 minutes with 4% (w/v) paraformaldehyde in PBS 1x and permeabilized for 2 minutes with 2% (w/v) NP-40 in PBS 1x. For the axoneme staining with antibody Ubdx2 (gift from D. Robinson, CNRS, Bordeaux, France) in Fig. 5, cells were fixed for 10 minutes in methanol 100% at –20°C. No permeabilization was needed after methanol fixation. Mitochondria were stained using anti-F1-ATPase antiserum (gift from D. Speijer, AMC, Amsterdam, The Netherlands) raised against the isolated mitochondrial ATPase of *Crithidia fasciculate* (dilution 1:200) (Fig. 2, 5B), or with an anti-Hsp60 antiserum raised against recombinantly expressed *T. brucei* Hsp60 fused to glutathione S-transferase (dilution 1:200) (Fig. 5A). Cells were washed with 1x PBS between incubations and embedded with Vectashield (Vector Labs, Emeryville, CA) supplemented with the DNA-intercalating agent DAPI for detection of DNA.

Flagellar pocket were detected by labeling of living cells with 10 mg/ml Fluorescein-conjugated tomato lectin (Vector Laboratories), as described (12).

Isolation of cytoskeleton

Cells were washed twice in lysis buffer (MOPS 100mM, pH 7.6) containing 1% Triton-X for 2 minutes. The resulting cytoskeletons were washed for 2 minutes in lysis buffer containing 0.1% Triton-X and finally in the same buffer without Triton-X. Then the cytoskeleton were fixed for 10 minutes with 4% (w/v) paraformaldehyde in PBS 1x and stained for immunofluorescence as for whole cells. No permeabilization was needed. All steps were done with cells dried on slides.

Immunoblot

To detect the Ty1 tagged TbMiX on immunoblot, we used a 1:20 dilution of the BB2 antibody. The others antibodies (EF1a, KDH and Cytochrome *c*) were diluted 1:10’000, 1:500 and 1:100, respectively. Detection was done with the SuperSignal West Femto Max Sensitivity Substrate from Pierce.

Results

Identification of TbMiX

To identify the MiX homologue of *T. brucei*, we did a BLAST analysis of the *T. brucei* genomic database using the MiX protein from *L. major* (LmMiX) as a template (Uboldi *et al*, submitted). By this way we identified the ORF Tb927.5.3040 encoding a 198 amino acids long polypeptide with a predicted molecular mass of 23kD. This protein was termed TbMiX. Orthologues of the protein are restricted to the *Kinetoplastidae*. TbMiX shows a high level of identity with its counterparts in *L. major* (72%) and *T. cruzi* (82%) (Fig. 1).

TbMiX shows no obvious known conserved domains. It has a single transmembrane helix in the N-terminal part (residues 26-48) of the protein. Interestingly the first nine amino acids are conserved in all MiX proteins and according to Uboldi *et al* may represent an unconventional mitochondrial targeting sequence.

TbMiX is an outer mitochondrial protein

MiX is colocalized with the mitochondrion in *L. major* (Uboldi et al, submitted). To determine its localization in *T. brucei*, we prepared a transgenic cell line which allows the expression of TbMiX carrying a Ty1-epitope tag at its C-terminal end (8). Immunofluorescence of the induced strain using an anti-epitope antibody shows a net-like staining that exactly colocalizes with the mitochondrion (Fig. 2A). Moreover, the mitochondrial localization of TbMiX was also confirmed by biochemical analysis. The tagged TbMiX is present in the same fraction than the mitochondrial marker, but cannot be detected in the cytosol (Fig. 2B). Thus these experiments show that TbMiX, identical to its leishmanial homologue, is localized in mitochondria.

To elucidate the intramitochondrial localization of TbMiX, we isolated mitochondria from cells expressing the tagged TbMiX and treated them with proteinase K. The following results were obtained: The signal of the tagged TbMiX was lost during proteinase K incubation, whereas cytochrome *c*, a protein of the mitochondrial intermembrane space, was protected from digestion. However when Triton-X is added to mitochondria and thus the outer membrane is disrupted, cytochrome *c* is completely digested (Fig. 2C). Because the Ty1 tag was added at the carboxyl terminus of TbMiX, these results show that the C-terminal part of

the protein is exposed to the cytosol. Thus these experiments prove that TbMiX is an integral protein of the outer mitochondrial membrane.

TbMiX is essential for normal growth

In *L. major*, heterogenous disruption of the LmMiX protein gene had strong effects on cellular morphology and weaker ones on mitochondrial organization and kinetoplasts segregation (Uboldi et al, submitted). Thus to analyze the function of TbMiX in *T. brucei*, we decided to make use of RNA interference (RNAi) (13). It should be mentioned here that the same analysis is not possible in *L. major* since it is lacking the RNAi machinery (14). Thus we established a cell line allowing tetracycline-inducible ablation of TbMiX. Interestingly, induction of RNAi causes a slow growth phenotype but did not completely stop growth (Fig. 3). This might be due to fact that RNAi does only remove 90-95% of the protein and that the small amount that remains is sufficient to support growth albeit at a lower rate.

TbMiX ablation leads to accumulation of cells with misplaced kDNA

The mitochondrial DNA of *T. brucei* is of one unit nature. It shows a distinct S-phase, that is coordinated with the nuclear S-phase (15). More precisely the kDNA replication is initiated just before the onset of the nuclear S-phase, and the segregation of mitochondrial DNAs occurs before mitosis. Because of the delay between kDNA and nuclear segregations, three defined cell cycle stages can be determined by analyzing Dapi-stained cells. These stages are defined by the numbers of kinetoplasts and nuclei and are termed 1K1N, 2K1N and 2K2N (K stands for kDNA and N for Nucleus) (Fig. 4B) (15, 16).

Because in *L. major*, LmMiX was shown to influence kinetoplast division (Uboldi et al, submitted), we decided to analyze the kDNA/nucleus configurations in cells ablated for TbMiX. Interestingly we observed a strong accumulation of abnormal configurations in these cells. In other words cells showing a pattern different from 1K1N, 2K1N and 2K2N accumulated and after 6 days of induction, reached approx. 50% of the total population (Fig. 4A).

The two most frequent aberrant kDNA/Nucleus configurations that have been described in *T. brucei* arise because the mitosis to cytokinesis checkpoint is missing in this organism (4, 17). This results in the appearance of cells containing one kDNA but lacking the nucleus (1K0N), also termed zoids, if mitosis is blocked (17), or in accumulation of cells with multiple kDNAs

and nuclei (xKxN) when cytokinesis is defective (4). However these two aberrant configurations represent only a small fraction of the ones observed in cells ablated for TbMiX. The additional aberrant configurations that are observed in these cells can generally be characterized by a misplacement of the kDNA (Fig. 4D). Thus cells with a 1K1N configuration, but where the kDNA is anterior to the nucleus, as in epimastigote cells (16), accumulate to a level of 13% after 4 days of induction. Furthermore we see the appearance of cells showing a KNK configuration, meaning that the single nucleus is surrounded by two kDNAs. These cells amount to 12% of the total population 5 days after induction. In a small fraction of the KNK cells, the nucleus is apparently able to divide, resulting in a KNNK configuration. Moreover, in addition to the cells with misplaced kDNAs, zoids accumulate continuously, reaching 16% of the total population after 10 days of induction. This is probably the result of ongoing cytokinesis in the cells with misplaced kDNAs. The same is probably also true for a special type of zoids that contain two kinetoplasts (2K0N). Thus cytokinesis does not appear to be affected when the kDNA is not correctly positioned. Finally, some cells showing 1K2N and multinucleated configurations are visible (approx. 5% each after 6 days of induction) (Fig. 4C, 4D). In summary these observations suggest that TbMiX is required to correctly position the kDNA in procyclic *T. brucei*.

To elucidate the consequences of the misplacement of the kDNA in cells lacking TbMiX might have, we analyzed other structures and organelles that are closely associated with the kDNA. These results showed that TbMiX depletion does not affect the association of the kDNA with the basal bodies, the flagellar pockets, the flagellar attachment zones nor the flagella itself (Fig. 5). Thus the tripartite attachment complex linking the kinetoplast to the basal body is not affected by TbMiX depletion.

TbMiX links the mitochondrion to cytoskeleton

The changes in position of the kDNA during the life cycle and the cell cycle are microtubule-mediated (2, 5). However the structural link between the subpellicular cytoskeleton and the kinetoplast region of the mitochondrion has not been found. Since TbMiX is present on the outer mitochondrial membrane, and since its depletion results in kDNA misplacement, we decided to check whether this protein could be involved in linking the mitochondrion to the cytoskeleton of *T. brucei.*

In order to do so, we isolated cytoskeleton of induced and uninduced cells expressing TbMiX containing a Ty1 epitope tag (8), and we co-stained the cells with BB2, the antibody recognizing the tag, and with an antiserum against the inner mitochondrial membrane-specific F1-ATPase. This experiment showed that some mitochondrial residues are still present after the cytoskeleton isolation, and that they are mainly co-localized with the tagged TbMiX (Fig. 6A). Moreover, isolation of cytoskeletons from the induced TbMiX RNAi strain revealed a strong reduction of the mitochondrial residues in comparison with the uninduced cells consistent with a weakening of the link between mitochondrion and cytoskeleton (Fig.6B). Thus, these results support the hypothesis that TbMiX might be required for establishing and maintaining the interaction between the mitochondrion and the cytoskeleton.

Discussion

Our results show that, in *T. brucei*, TbMiX, a protein specific to the *Kinetoplastidae*, is present on the outer mitochondrial membrane. Furthermore we show that the protein is essential for normal growth and for the correct positioning of the kDNA, but not required for cytokinesis. Finally we provide evidence suggesting that TbMiX may link the mitochondrion to the cytoskeleton.

The microtubular cytoskeleton of eukaryotic cells has several different functions. It is not only responsible of the cell shape, but also builds the flagella required for cell movement. Furthermore it plays an important role in intracellular transport and cell division. In *T. brucei*, the cytoskeleton is mainly involved in maintenance of the cell shape. It forms a subpellicullar corset of parallel microtubules underlying the plasma membrane (18). Only a small flask-shaped invagination of the plasma membrane, termed the flagellar pocket, lacks this microtubule mantle. Thus endocytosis and exocytosis are restricted to this pocket, from which the flagellum exits the cell body (18). During cytokinesis, the cell shape has to be maintained. The newly formed microtubules are therefore laterally added between the old ones (1).

The highly polarized cytoskeleton of *T. brucei* is important for the positioning of most organelles (5). Moreover, the changes in the position of the kDNA during the life cycle are also microtubule-mediated (2). Based on this we would expect a link between the cytoskeleton and the kinetoplast or the mitochondrion to exist. Since TbMiX is evenly distributed on the outer mitochondrial membrane, with the main part of the protein exposed to the cytosol (Fig. 2), it is a candidate protein to be involved in this linkage. Furthermore the effect TbMiX depletion has on kDNA positioning (Fig. 4) and on the attachment of the mitochondrion to the detergent-extracted cytoskeletons (Fig. 6) supports its role in mitochondrion-cytoskeleton interactions. Thus, if the mitochondrion is no more firmly attached to the cytoskeleton, this is expected to influence the position of the kDNAs and may result in the displacements observed in the TbMiX ablated cells.

Interestingly, TbMiX depletion does not cause any global changes in the mitochondrial morphology (Fig. 5). An explanation could be that the mitochondrion in procyclic cells is quite big and takes up a large space in the cell. Thus, even if the binding with the cytoskeleton is disrupted, this may not immediately result in morphological changes.

Inhibition of microtubule dynamics by addition of anti-microtubule drug rhizoxin leads to zoid formation, similar to what is observed in the TbMiX RNAi cell line. However in this case, a disturbed axis of the cleavage furrow appears to be responsible for zoid formation (5), whereas in TbMiX ablated cells, zoid accumulation is probably directly due to the misplacement of the kDNA.

An interesting aspect of the phenotype observed in TbMiX RNAi, is that the positioning of the kDNA anterior to the nucleus is characteristic of the epimastigote forms found in the salivary glands of the tsetse fly. Thus it could be that TbMiX depletion triggers the differentiation into epimastigote forms. Further studies such as staining of TbMiX-depleted cells with epimastigote specific marker are required to clarify this point.

In *T. brucei* some cell cycle checkpoints are missing (4). Thus mitosis is completely dissociated from cytokinesis (19). Cytokinesis depends on the flagellar attachment zone (20), a structure closely associated with the flagellum. The basal body, the base of the flagellum, is itself linked to the kinetoplast DNA by the tripartite attachment complex (5, 6). Thus kDNA segregation is tightly linked to cytokinesis. It has been proposed that, since the mitosis is not coupled to cytokinesis, kDNA segregation may act as a checkpoint (4). In TbMiX RNAi cells the kDNA is segregated albeit not to the correct positions. Thus the putative checkpoints would not be triggered and the cytokinesis will continue.

In conclusion the mispositioning of the kDNA in the absence of TbMiX and the putative involvement of TbMiX in cytoskeleton/mitochondrion interactions is highly interesting and may help in the understanding of cell cycle regulation in *T. brucei*.

Figure legends

Fig. 1: Identification of the MiX homologue in *T. brucei.* Amino acids alignment of MiX homologues of *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major.* The sequences were aligned using the CLUSTALW program with default parameters. Strictly conserved residues and conservative replacements are shown in black and grey boxes, respectively. Putative mitochondrial targeting sequences (MTS) and the putative transmembrane (TM) domains of TbMiX are underlined in black and grey, respectively.

Fig. 2: TbMiX is localized on the outer mitochondrial membrane. A) Normarski picture and double-immunofluorescence of cells expressing TbMiX carrying a Ty1-tag at its carboxyl terminus under the control of the tetracycline-inducible procyclin promoter (- tetra, + tetra). The cells were stained with Dapi for DNA (blue), with an antiserum against a subunit of the ATPase as a mitochondrial marker (green) and with the BB2 monoclonal antibody recognizing the Ty1-tag (red). A merged staining of the mitochondrial marker with the tagged TbMiX is shown for induced cells. B) Immunoblot of total cells and cellular extracts of induced and uninduced cells (+ tetra, - tetra) expressing Ty1-tagged TbMiX. Crude cytosolic (cyto.) and crude mitochondrial (mito.) extracts of induced cells were isolated by treatment with 0.03% digitonin. Elongation factor 1a (EF1a) and α-ketoglutarate dehydrogenase (KDH) serve as cytosolic and mitochondrial markers, respectively. The Ef1a staining in the mitochondrial fraction indicates that some cells were not lysed during the digitonin treatment. C) Immunoblot of a protease protection assay of crude mitochondrial extracts obtained by digitonin treatment of cells expressing Ty-1 tagged TbMiX. Cytochrome *c* was used as marker for the intermembrane space of mitochondria. Triton-X-100 was added to destroy all membranes in order to show that cytochorme *c* is sensitive to protease.

Fig. 3: TbMiX is essential for normal growth of procyclic *T. brucei.* A) Growth curve of a TbMiX RNAi cell line in the presence and in absence of tetracycline (+ tetra, - tetra). B) Northern blot of TbMiX mRNA during induction of RNAi. The times of sampling are indicated. The rRNAs visualized by ethidium bromide staining (EtBr) in the lower panel serve as a loading control.

Fig. 4: Inhibition of TbMiX leads to accumulation of abnormal cells with misplaced kDNA. A) Kinetic of appearance of the different kDNA/Nuclei configurations of Dapi-stained cells during TbMiX RNAi induction. The graph represents the percentages of cells containing the indicated number of nuclei and kDNAs (1K1N, 2K1N, 2K2N, Abnormal and Dead cells). n > 1000 cells. B) Examples of Dapi-stained cells with normal kDNA/Nuclei configurations seen in wild-type cells (1K1N, 2K1N, 2K2N). Nomarski (left panel) and Dapi staining (right panel) are shown. C) Kinetic of appearance of the abnormal kDNA/Nuclei configurations indicated in A). The graph represents the percentages of cells of the total population containing the indicated number of nuclei and kDNAs (Inverted 1K1N, KNK, KNNK, 1K0N, 2K0N, 1K2N and xKxN). D) Examples for the abnormal kDNA/Nuclei configurations described in C).

Fig. 5: Depletion of TbMiX shows results in mispositioning of the kDNA and its associated structures. Immunofluorescence of uninduced and induced TbMiX cells. For uninduced cells a 2K1N cell and for induced cells a KNK configuration is shown. Cells were stained with different antibodies and dyes to visualize the mitochondrion (Hsp60), the newly tyrosinated α-tubulin including the basal bodies (YL1/2), the flagellar pocket (Tomato lectin), the flagellar attachment zone (FAZ) and the flagellum (Axoneme). All cells were co-stained with Dapi. Nomarski pictures are shown on the left of each panel.

Fig. 6: A) Double-immunofluorescence on Triton-X-100 extracted cytoskeletons of uninduced and induced cells expressing the TbMiX carrying a Ty1-tag at its carboxyl terminus under the control of the tetracycline-inducible promoter. The cells were stained with an antiserum against a mitochondrial membrane-specific F1-ATPase as mitochondrial marker (green), and with the monoclonal antibody BB2 recognizing the Ty1-tag (red). A merged staining of the mitochondrial marker and of TbMiX is shown for induced cells. Nomarski pictures of the stained cytoskeleton are also shown. B) Immunofluorescence on isolated cytoskeleton of uninduced and induced TbMiX RNAi cells, using the F1-ATPase antiserum. Nomarski pictures of the stained cytoskeleton are also shown.

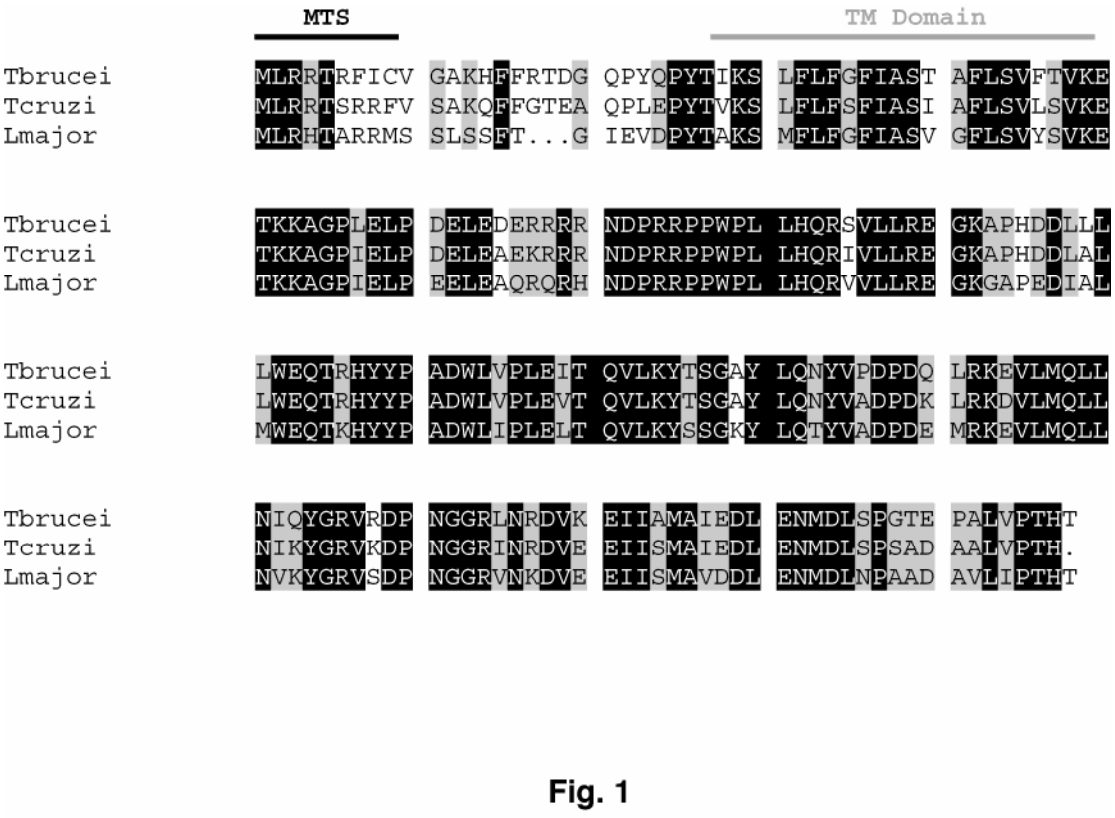
MTS
TM Domain
Tbrucei MLRRTRFICV GAKHFFRTDG QPYQPYTIKS LFLFGFIAST AFLSVFTVKE
Tcruzi MLRRTSRRFV SAKQFFGTEA QPLEPYTVKS LFLFSFIASI AFLSVLSVKE
Lmajor MLRHTARRMS SLSSFT...G IEVDPYTAKS MFLFGFIASV GFLSVYSVKE
Tbrucei TKKAGPLELP DELEDERRRR NDPRRPPWPL LHQRSVLLRE GKAPHDDLLL
Tcruzi TKKAGPIELP DELEAEKRRR NDPRRPPWPL LHQRIVLLRE GKAPHDDLAL
Lmajor TKKAGPIELP EELEAQRQRH NDPRRPPWPL LHQRVVLLRE GKGAPEDIAL
Tbrucei LWEQTRHYYP ADWLVPLEIT QVLKYTSGAY LQNYVPDPDQ LRKEVLMQLL
Tcruzi LWEQTRHYYP ADWLVPLEVT QVLKYTSGAY LQNYVADPDK LRKDVLMQLL
Lmajor MWEQTKHYYP ADWLIPLELT QVLKYSSGKY LQTYVADPDE MRKEVLMQLL
Tbrucei NIQYGRVRDP NGGRLNRDVK EIIAMAIEDL ENMDLSPGTE PALVPTHT
Tcruzi NIKYGRVKDP NGGRINRDVE EIISMAIEDL ENMDLSPSAD AALVPTH.
Lmajor NVKYGRVSDP NGGRVNKDVE EIISMAVDDL ENMDLNPAAD AVLIPTHT

Fig. 1

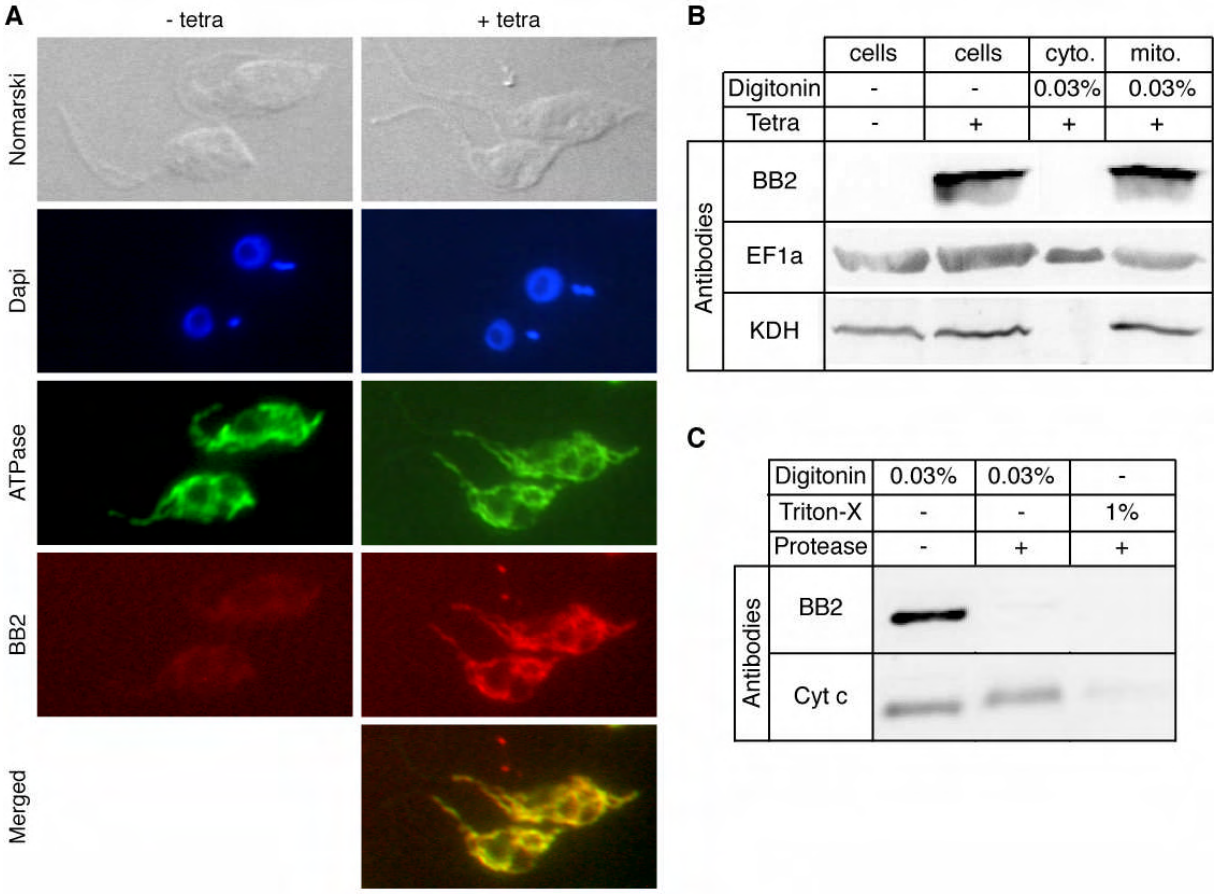
A
- tetra
+ tetra
Nomarski
Dapi
ATPase
BB2
Merged
B
cells
cells
cyto.
mito.
Digitonin
-
-
0.03%
0.03%
Tetra
-
+
+
+
Antibodies
BB2
EF1a
KDH
C
Digitonin
0.03%
0.03%
-
Triton-X
-
-
1%
Protease
-
+
+
Antibodies
BB2
Cyt c

Fig. 2

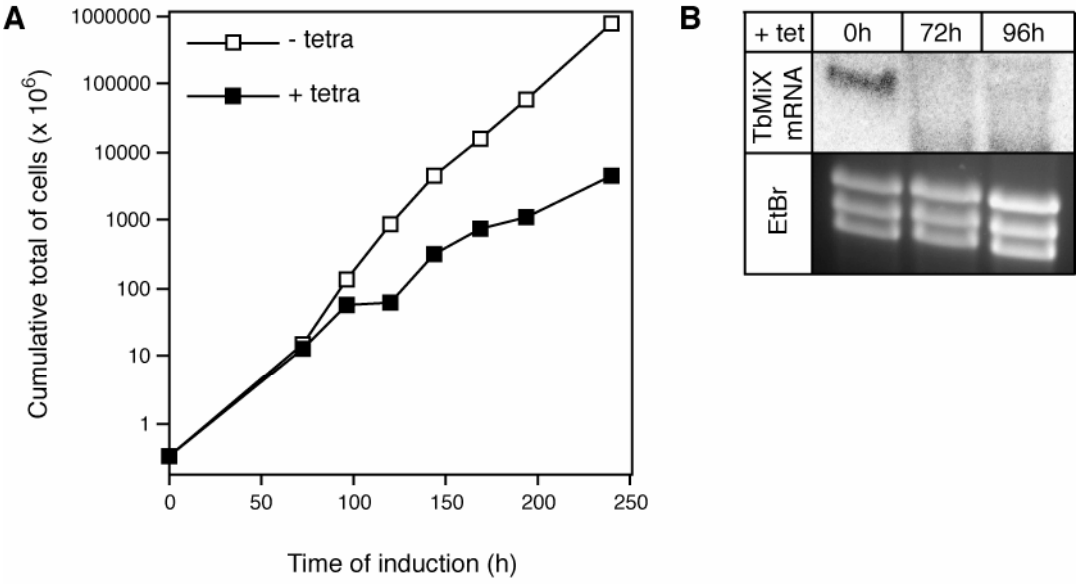
A
1000000
100000
10000
1000
100
10
1
- tetra
+ tetra
Cumulative total of cells (x 10^6)
0
50
100
150
200
250
Time of induction (h)
B
+ tet
0h
72h
96h
TbMiX mRNA
EtBr

Fig. 3

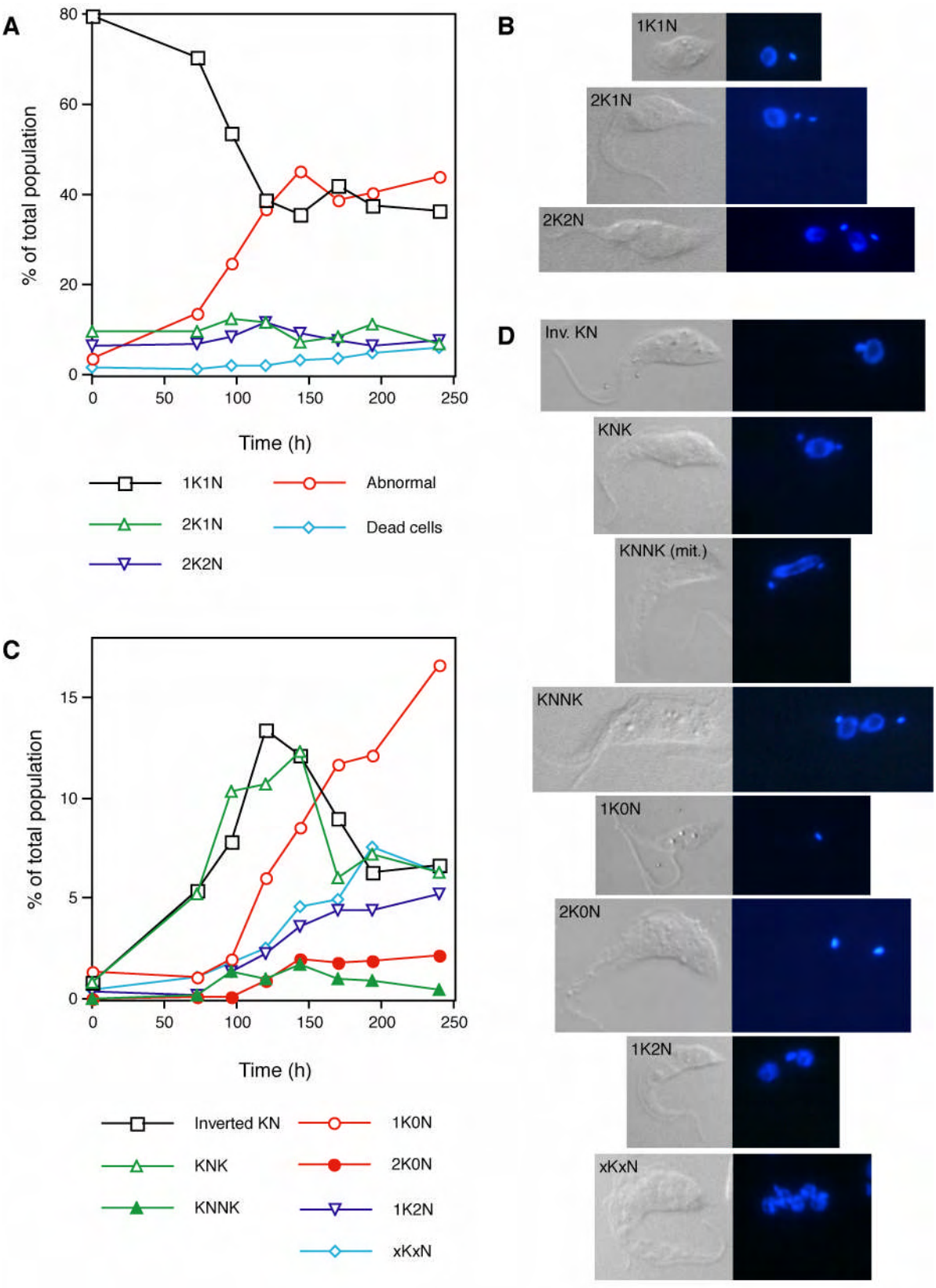
A
% of total population
Time (h)
1K1N
2K1N
2K2N
Abnormal
Dead cells
B
1K1N
2K1N
2K2N
C
% of total population
Time (h)
Inverted KN
KNK
KNNK
1K0N
2K0N
1K2N
xKxN
D
Inv. KN
KNK
KNNK (mit.)
KNNK
1K0N
2K0N
1K2N
xKxN

Fig. 4

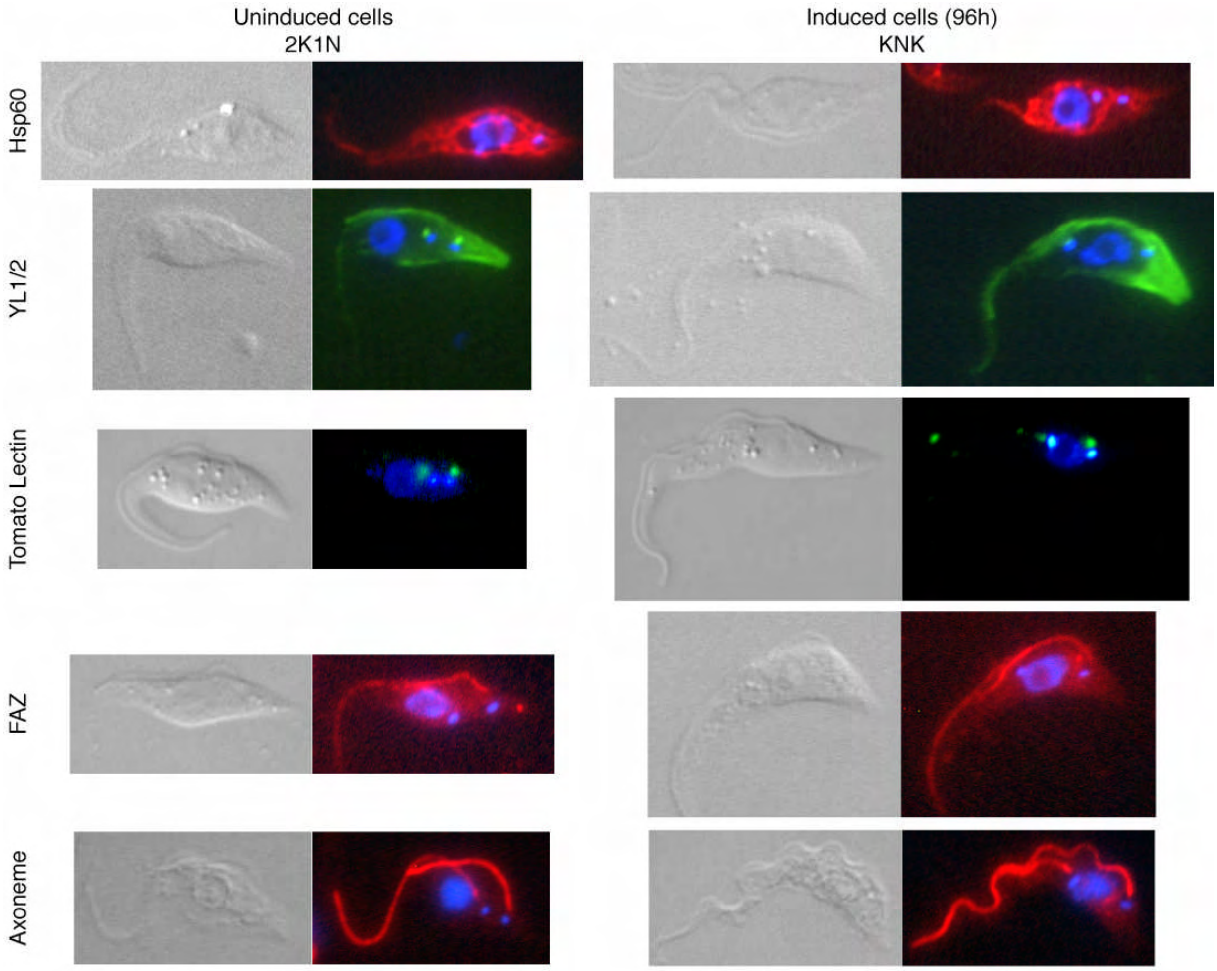
Uninduced cells
2K1N
Induced cells (96h)
KNK
Hsp60
YL1/2
Tomato Lectin
FAZ
Axoneme

Fig. 5

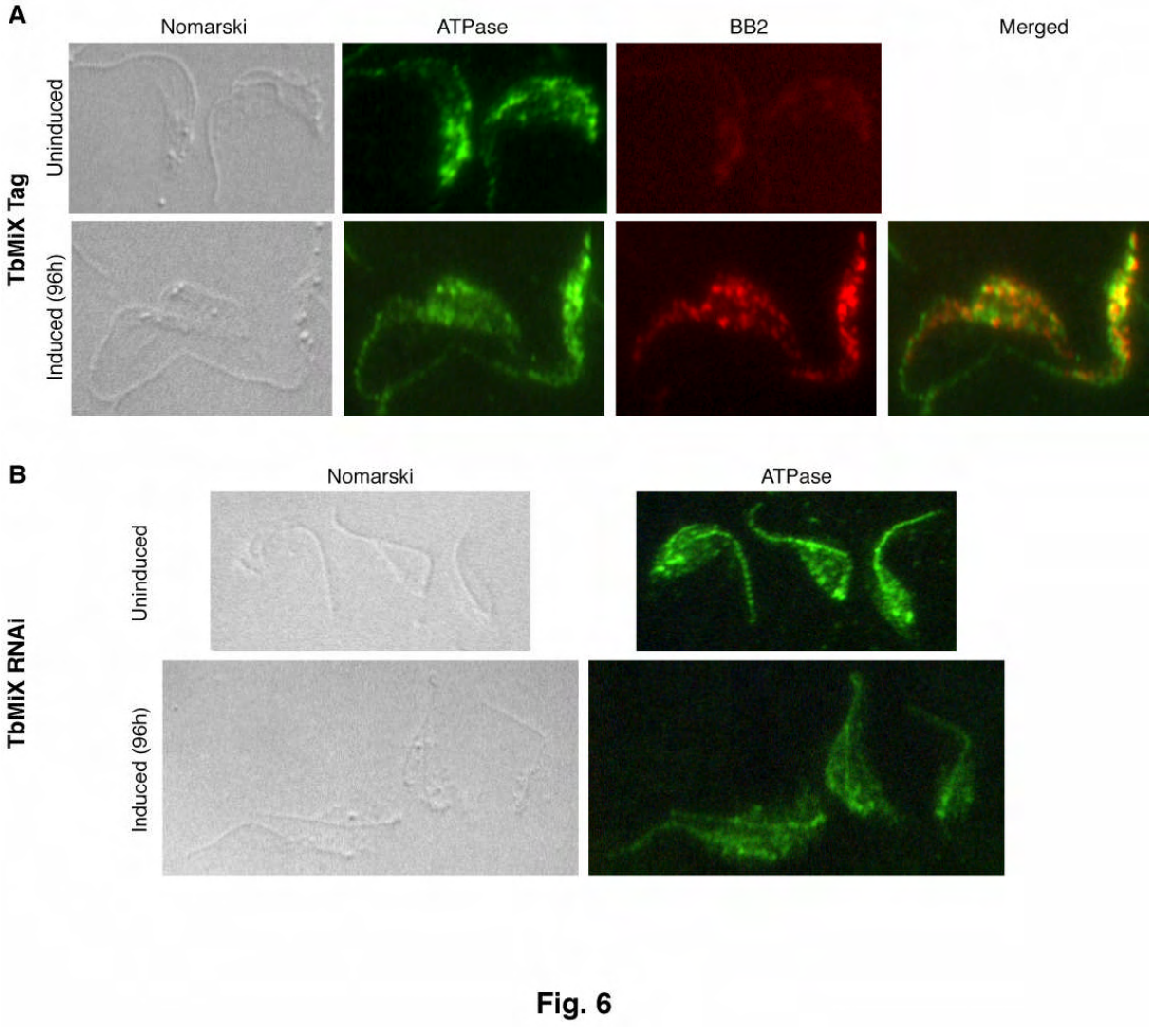
A
Nomarski
ATPase
BB2
Merged
TbMiX Tag
Uninduced
Induced (96h)
B
Nomarski
ATPase
TbMiX RNAi
Uninduced
Induced (96h)

Fig. 6

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Crausaz Esseiva A.*, **Chanez A.-L.***, Bochud-Allemann N. Martinou J.-C., Hemphill A., Schneider A. *Temporal dissection of Bax-induced events leading to fission of the single mitochondrion in Trypanosoma brucei* (2004) EMBO Reports **5**, 3, 268-273
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Gentle I.E., Perry A.J., Alcock F.H., Liki V.A., Dolezal P., Ng E.T., McConnville M., Naderer T., **Chanez A.-L.**, Charrière F., Aschinger C., Schneider A., Tokatlidis K., Lithgow T. *Conserved motifs reveal details of ancestry and structure in the small TIM chaperones of the mitochondrial intermembrane space*. (2007) Submitted

Schneider A., Bouzaidi-Tiali N., **Chanez A.-L.**, Bulliard L. *ATP production in isolated mitochondria of procyclic Trypanosoma brucei*. Submitted

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November 05	PhD Meeting of Swiss Society of Tropical Medicine and Parasitology Monte Verita/Ascona (Switzerland)
January 05	Annual Swiss Trypanosomatid Meeting Leysin (Switzerland)
September 04	Molecular Parasitology Meeting Woods Hole, MA (USA)
September 04	MitEuro plenary meeting (European meeting for mitochondria) Aussois (France)
January 04	Annual Swiss Trypanosomatid Meeting Leysin (Switzerland) Winner of the best presentation
October 03	PhD Meeting of Swiss Society of Tropical Medicine and Parasitology Münchenwiller/Morat (Switzerland)

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English	Very good knowledge, fluent speaking and writing
German	Good knowledge

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