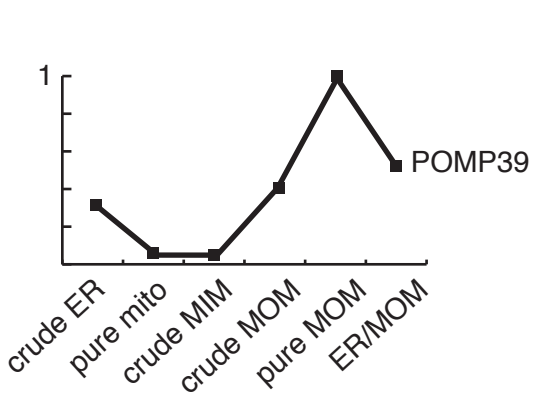
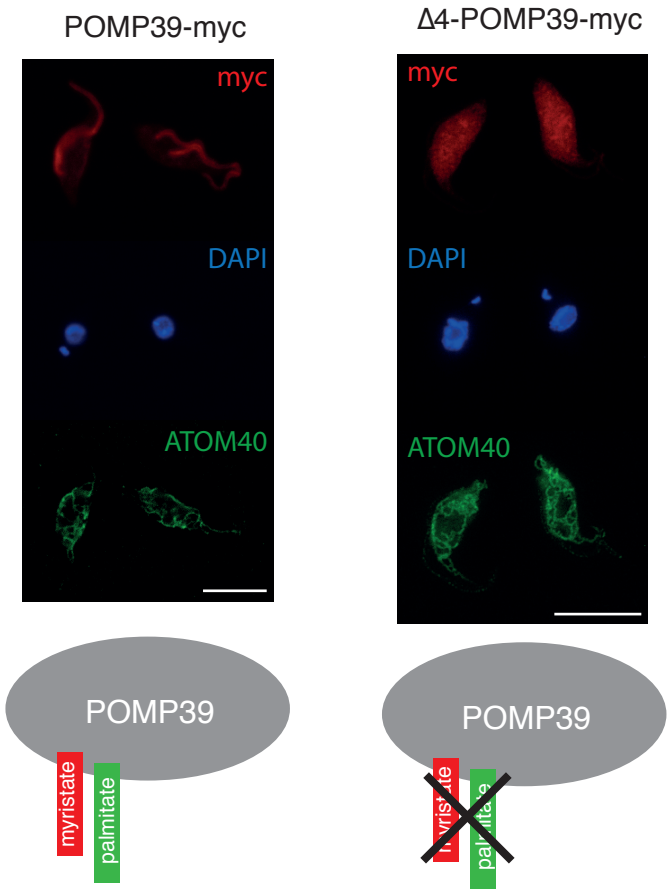
***Graphical Abstract**



POMP39 is a protein dually localized to the mitochondrial outer membrane (MOM) and the flagellum of *T. brucei*. Its sub-organellar localization depends on acylation.



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HIGHLIGHTS

- A protein of the mitochondrial outer membrane (MOM) proteome of African trypanosomes is dually localized
- The protein localizes to both the MOM and to the flagellum
- The sub-organellar localization depends probably on dual acylation
- The localization of the protein was also investigated in the disease-causing bloodstream form of the parasite

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TITLE:

A component of the mitochondrial outer membrane proteome of *T. brucei* probably contains covalent bound fatty acids.

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ABSTRACT:

A subclass of eukaryotic proteins is subject to modification with fatty acids, the most common of which are palmitic and myristic acid. Protein acylation allows association with cellular membranes in the absence of transmembrane domains. Here we examine POMP39, a protein previously described to be **p**resent in the **o**uter **m**itochondrial membrane **p**roteome (POMP) of the protozoan parasite *Trypanosoma brucei.* POMP39 lacks canonical transmembrane domains, but is likely both myristoylated and palmitoylated on its N-terminus. Interestingly, the protein is also dually localized on the surface of the mitochondrion as well as in the flagellum of both insect-stage and the bloodstream form of the parasites. Upon abolishing of global protein acylation or mutation of the myristoylation site POMP39 relocates to the cytosol. RNAi-mediated ablation of the protein neither causes a growth phenotype in insect-stage nor bloodstream form trypanosomes.

KEYWORDS:

Mitochondrial outer membrane proteome, African trypanosomes, protein acylation, myristoylation/palmitoylation, dual localization of proteins, protein targeting

Note: Supplementary data associated with this article

1. INTRODUCTION

African trypanosomes are unicellular, flagellated eukaryotic parasites that are transmitted by the Tsetse fly during a blood meal. During their complex life cycle trypanosomes switch their surface coat and modulate many cellular functions. As bloodstream forms (BSF) trypanosomes cause sleeping sickness in humans and nagana in cattle. Besides their clinical importance trypanosomes are also excellent model systems to study basic biological processes. One reason for this is that, unlike the common model organisms mouse, flies, worms and yeast, which belong to the eukaryotic supergroup of the *Opisthokonta*, trypanosomes are representatives of the supergroup of the *Excavata* (Simpson and Roger, 2004). This unique position in the eukaryotic evolutionary tree results in many special features in their biology, many of which concern mitochondrial biogenesis. As a consequence, African trypanosomes have recently been subjected to sub-cellular and sub-organellar proteomics approaches scrutinizing the protein inventory of mitochondria and their membranes (Acestor et al., 2009; Niemann et al., 2013; Panigrahi et al., 2009), the mitochondrial ribosomes (Zikova et al., 2009) and the respiratory chain (Acestor et al., 2009). Moreover, the composition of the flagellum has also been analyzed in detail (Broadhead et al., 2006; Oberholzer et al., 2011). These comprehensive proteomics approaches have greatly increased the knowledge about the sub-cellular localization of a large fraction of trypanosomal proteins and resulted in an increasing number of proteins that have been detected in two or more cellular compartments. However, it is often unclear whether this reflects cross-contamination that is inherent in many biochemical purifications or whether these proteins are genuinely dually localized and moonlight in an alternative sub-cellular context (Collingridge et al., 2010). In a recent study we have raised the protein inventory of the mitochondrial outer membrane (MOM) of *T. brucei* by combining sub-organellar fractionation with correlated protein abundance profiles and statistical analysis (Niemann et al., 2013). Out of the 82 MOM proteins identified, only 30 have predicted transmembrane helices and five belong to the ß-barrel protein family (Niemann et al., 2013). Thus, 47 MOM proteins have no canonical membrane-spanning domain, leading to the question as to how these proteins associate with the MOM.

Aside from hydrophobic α-helical domains, protein modifications with fatty acids provide a mechanism to anchor proteins to membranes. There are many different ways of how proteins can be acylated; among them the covalent modification of an N-terminal glycine residue with myristate (a 14-carbon chain fatty acid) probably represents the best-characterized protein fatty acid modification. Proteins with the N-terminal consensus sequence MGXXXS/T (Maurer-Stroh et al., 2010a; Maurer-Stroh et al., 2010b; Towler et al., 1988) are subject to this irreversible modification, which is carried out by the enzyme myristoyl-CoA:protein *N*-myristoyltransferase (NMT) (Bhatnager et al., 1999; Farazi et al., 2001), usually in a co-translational fashion (Martin et al., 2011). Whereas myristoylation of a protein increases its hydrophobicity and thus its membrane affinity, this single acylation is considered insufficient for anchoring a protein stably to a membrane (Peitzsch and McLaughlin, 1993). Monomyristoylated proteins can thus only transiently associate with membranes.

Reversible S-acylation represents another way to lipidate a protein. S-acylation consists of the modification of a cysteine side chain with a fatty acid, most frequently palmitate, the carbon chain of which is longer than myristate. Palmitoylation is catalyzed by the enzyme palmitoyl-acyltransferase (PAT). Multiple PATs and target proteins have been described, e.g. 23 PATs and >250 targets in mammalian cells and 7 PATs and >50 targets in yeast (for reviews see e.g. (Greaves and Chamberlain, 2011; Hannoush and Sun, 2010; Mitchell et al., 2006; Nadolski and Linder, 2007). Moreover, thioester-bound fatty acids including palmitate can be removed by the activity of acyl protein thioesterase (APT) (Duncan and Gilman, 2002; Zeidman et al., 2009). Thus, in contrast to the co-translational and irreversible myristoylation that generally is mediated by a single NMT, the palmitoylation is reversible, occurs post-translational and is catalyzed by multiple PATs. Many proteins are dually acylated, which allows them to stably associate membranes (Nadolski and Linder, 2007). The interplay of myristoylation and palmitoylation and the resulting implications for protein routing, trafficking and dynamic regulation of protein localization are poorly understood. However, the kinetic membrane-trapping model (Shahinian and Silvius, 1995) provides a mechanism of how cycles of acylation-deacylation by PATs and thioesterases might regulate sub-cellular localization of fatty acid modified proteins (Goodwin et al., 2005; Rocks et al., 2005; Shahinian and Silvius, 1995).

In kinetoplastids, the dual acylation with myristate and palmitate has been linked to flagellar localization of proteins (Emmer et al., 2009; Liu et al., 2010, for a review of acylation in trypansomatids see Goldston et al., 2014). Another study shows that dual acylation is associated with the localization to the endomembrane system (Mills et al., 2007). Here we present evidence for the acylation of a protein that is dually localized to both, the MOM and the flagellum of *T. brucei*. The membrane-association of the polypeptide termed POMP39 (**p**resent on the mitochondrial **o**uter **m**embrane **p**roteome #39; Niemann et al., 2013) likely depends on the dual acylation at the N-terminus of the protein. In summary our result suggest that protein acylation represents a versatile mechanism to allow association with different membranes within the same cell.

2. MATERIAL AND METHODS

2.1 Cell lines

Insect-stage (procyclic form, PCF) *T. brucei* cell lines were generated using strain 29-13 (Wirtz et al., 1999) and were grown at 27°C in SDM79 (Brun and Schönenberger, 1979) supplemented with 10% (w/v) fetal calf serum. Transgenic BSF *T. brucei* are based on the single marker strain (Wirtz et al., 1999). BSF cultures were grown in HMI-9 (Hirumi and Hirumi, 1989) at 37°C and 5% CO_2 atmosphere. POMP39 was tagged with a triple c-myc epitope at both the N- and C-terminus using a pLew-100 (Wirtz et al., 1999) derivative and puromycin as selection marker. The RNAi is directed against the ORF Tb927.8.2070, but due to the sequence similarities (see SupFig.1 and SupFig.2) all of the four putative isoforms are simultaneously affected by the RNAi. Collectively we refer to *POMP39 RNAi* throughout the manuscript. The RNAi construct was generated based on a pLew-100 stem-loop construct

(Wirtz et al., 1999) with blasticidin as selection marker using a 433bp-insert (nucleotides 158-591 of Tb927.8.2070). The TbNMT RNAi cell line was described by Price et al., 2003. Transfection of the constructs was done as described (McCulloch et al., 2004).

2.2 Subcellular Fractionation and Western Blot

Preparation of mitochondrial vesicles at isotonic conditions was carried out as described in detail by Schneider et al., 2007a. Digitonin fractionation was performed as specified in Schneider et al., 2007b. Carbonate extraction was done as demonstrated in Fujiki et al., 1982 but using a Beckman Airfuge for pelleting at 100.000g for 20min. Protein samples were analyzed using standard procedures for SDS-PAGE and western blotting. Polyclonal antisera from rabbits were diluted in phosphate buffered saline (1xPBS) containing 0.1% (v/v) Tween and 3% (w/v) milk powder as follows: VDAC (voltage-dependent anion channel) 1:1000, cyC (cytochrome C) 1:100, cyC1 (cytochrome C1) 1:1000, ATOM40 (archaic translocase of the MOM) 1:10.000, LDH (dihydrolipoamid-dehydrogenase, gift of L. Krauth-Siegel, Heidelberg) 1:10.000, mHSP70 (mitochondrial heat shock protein 70, gift of Rob Jensen, Baltimore). Commercially available monoclonal mouse antibodies anti EF1a (eukaryotic elongation factor 1a; Upstate/Merck Millipore) and anti c-myc (Santa Cruz Biotech) were diluted 1:10.000 and 1:1000. Western blots were visualized with the Odyssey Clx infrared imaging system from Li-Cor Biosciences.

2.3 Immunofluorescence

Immunofluorescence was carried out according to standard protocols. Briefly, ca. 0.5-1x10^7 PCF or BSF cells were harvested, washed twice in 1xPBS and adhered to the glass slide for 10min. The cells were then fixed in 1xPBS/4%(w/v) paraformaldehyde for 10min and permeabilized in 1xPBS/2% (w/v) Triton-x-100 for 10min. The slides were incubated with the primary and secondary antibodies for one hour each. The antibody incubations were each followed by five two-minute washes with 1xPBS. All steps were carried out in a wet chamber at room temperature in the dark. Finally, the slides were submerged in ice-cold methanol for three min and air-dried. Slides were mounted with DAPI-containing (1.5µg/µL) Vectashield (Vector Laboratories) and imaged with a Leica DMI6000 B microscope equipped with a modified light source to visualize the infrared spectrum. Antisera/antibodies were diluted in 1xPBS as follows: c-myc 1:100; ATOM40 1:1000; FITC conjugated to goat anti-rabbit 1:100 (Sigma); IRDye 680LT conjugated to goat anti-mouse 1:100 (Li-Cor Biosciences).

2.4 Northern Blot

To ascertain the tetracycline-inducible (tet) RNAi-mediated gene ablation of POMP39, RNA was isolated from uninduced cells and 48hrs post-induction. Northern blot was performed as described (Tan et al., 2002).

3. RESULTS

3.1 POMP39 is likely myristoylated and palmitoylated.

The protein POMP39 was found as component of the MOM proteome in PCF trypanosomes that combined biochemical fractionation with quantitative mass spectrometry (Niemann et al., 2013). It is encoded by four nearly identical ORFs (Tb927.8.2070, Tb927.8.2260, Tb927.8.2270, Tb927.8.2280), which differ by 2-7 amino acids among each other (see SupFig. 1). We thus refer to "POMP39" for all four potential isoforms. BLAST analysis shows that the protein has an orthologue in *T. brucei gambiense* and *T. evansi*, but not in other trypanosomatids or in any other species. Sequence analysis with HHPred (Biegert et al., 2006) suggests that POMP39 may have a region sharing vague similarity with a phosphodiesterase domain although most amino acids are missing or replaced in the predicted active site, which suggests that it cannot be active. POMP39 has neither a predicted transmembrane helix nor a signal anchor. The polypeptide is strongly overexpressed in the BSF, as evident from proteomics studies (Bridges et al., 2008; Gunasekera et al., 2012).

Bioinformatics analyses suggest that POMP39 is dually acylated (Fig. 1A). According to the NMT algorithm (http://mendel.imp.ac.at/myristate/), which predicts myristoylation sites, the glycine residue on the second position (G2) is subject to myristoylation with a high level of confidence (probability of false positive 0.03%). The cysteine residue on position three (C3) is a predicted palmitoylation site with ≥95% confidence level when analyzed using the CSS-Palm algorithm (Ren et al., 2008). Consistent with this prediction, POMP39 was identified as one of 124 proteins enriched by streptavidin chromatography following an acyl-biotin exchange-reaction of whole cell lysates (Emmer et al., 2011). Notably, this streptavidin-capture of POMP39 was hydroxylamine-sensitive. This substantiates the presence of an S-acylation on the POMP39 polypeptide. Thus, POMP39 is likely one of the <2% proteins of the ca. 7500 proteins in *T. brucei* that is palmitoylated.

To verify these findings, cell lines expressing three epitope-tagged variants of POMP39 were generated (Fig. 1B). The protein was tagged with the triple c-myc epitope on its N- (myc-POMP39) and its C-terminus (POMP39-myc). To abolish both fatty acid modifications, a variant of the C-terminally tagged POMP39 was generated lacking the first four amino acids including both putative acylation sites (∆4-POMP39-myc). Immunoblots of whole cell extracts resolved by SDS-PAGE from cell lines expressing the three tagged proteins revealed that the C-terminally tagged version migrates faster than the N-terminally tagged protein (Fig. 1B). Moreover, the deletion mutant shows the same mobility as the N-terminally tagged variant (Fig. 1B). Since acylated proteins are known to show an increased electrophoretic mobility on SDS PAGE (Lodge et al., 1997), the faster migrating c-terminally tagged POMP39 is likely acylated. In such a scenario the other two tagged POMP39 variants would not be acylated since the acylation site is masked by the myc-tag in myc-POMP39 or absent in ∆4-POMP39-myc.

It has previously been shown that N-terminal myristoyl-transferase (TbNMT) is responsible for essentially all protein myristoylation in *T. brucei* (Price et al., 2003). In order to directly investigate the putative myristoylation of POMP39 we produced an RNAi cell line allowing inducible ablation of TbNMT with simultaneous expression of C-terminally tagged POMP39 (POMP39-myc). In

this cell line, the addition of tetracycline triggers both, the expression of POMP39-myc as well as the ablation of TbNMT. As expected, the ablation of TbNMT leads to a growth arrest after around >90hrs of induction and ultimately to the death of the parasites (Fig. 1C) (see Price et al., 2003). During induction samples of digitonin extracts of total cells were collected and analyzed by SDS-PAGE. The result in Fig. 1C shows that concomitant with the appearance of the growth phenotype the electrophoretic mobility of POMP39-myc changes towards slower migration, which, as shown in Fig. 1B, is consistent with the lack of acylation. The fact that the migration of the protein in induced cells essentially matches the one observed for Δ4-POMP39-myc protein (Fig. 1B) suggests that it has lost both acyl groups. TbNMT is only essential for myristoylation but not for palmitoylation, suggesting that in the absence of the myristate the palmitoyl-group gets rapidly turned over or is not attached in the first place. The increase in the amount of the tagged POMP39 is due to ongoing induction of the expression of the tagged POMP39. The altered electrophoretic mobility upon TbNMT knockdown thus is indicative of the presence of the myristoylation of POMP39.

Carbonate extraction at high pH disrupts the interaction between membranes and peripherally associated proteins, and therefore can be used to distinguish them from membrane-integral proteins (Fujiki et al., 1982). POMP39 lacks predicted transmembrane domains and therefore would be expected to behave as a soluble protein in a carbonate extraction experiment, despite the presence of the myristoylation — which alone would not be sufficient to confer membrane integrity. However, in case POMP39 is dually acylated it would be expected to behave like a membrane-integral protein and should be recovered in the pellet. In order to test the acylation status of POMP39, mitochondrial vesicles from cells expressing POMP39-myc were isolated at isotonic conditions to keep the MOM intact (Schneider et al., 2007a) and subjected to carbonate extraction. Fig. 1D shows that POMP39-myc behaves like the membrane-integral protein VDAC (voltage-dependent ion channel), whereas the peripheral membrane protein cyC (cytochrome c) was exclusively recovered in the soluble fraction. The carbonate extraction thus indicates the presence of an additional acylation of POMP39 other than the putative myristoylation. Collectively, the data shown in Fig. 1 imply that POMP39 is dually acylated, namely probably myristoylated at position G2 and most likely palmitoylated at position C3.

3.2 POMP39 localizes to mitochondria and flagella

POMP39 was originally identified as a component of the MOM proteome. The strategy to identify MOM proteins was based on protein abundance profiling using mitochondrial sub-fractions. In combination with statistical analysis this allowed us to define a cluster of proteins that are localized to the MOM with high confidence (Niemann et al., 2013). Proteins that are localized to more than one compartment would in this study also be scored as MOM proteins, provided that the “second” localization would be in a fraction that was not included in our analysis. Thus, in order to determine the intracellular localization of POMP39 in more detail we employed a cell fractionation protocol (Fig. 2A upper panel). Briefly, the cells were lysed using nitrogen cavitation (WC). The crude cytosol (SN1) was separated from the

membranous fraction (P1) and upon differential centrifugation crude mitochondria were obtained (crude mito). In a last step, the crude mitochondrial fraction was purified by using a discontinuous nycodenz gradient to obtain pure mitochondria ("pure mito", see detailed description in Schneider et al., 2007a). Western blots of the fractionation show that mitochondrial proteins are most enriched in the "pure mito" fraction, as is evident by comparing the mitochondrial marker proteins ATOM40, cy C, cy C1 and mHSP70, which localize to the four different mitochondrial sub-compartments OM, IMS (intermembrane space), IM (inner membrane) and matrix (Fig. 2A upper panel, lane "pure mitos"). The cytosolic marker eukaryotic elongation factor 1a (EF1a) is most enriched in the cytosol (SN1) and is depleted in the "pure mito" fraction (Fig. 2A upper panel). POMP39-myc is not enriched in any particular fraction and thus behaves different from both mitochondrial and cytosolic markers. This strongly suggests that POMP39 is present in mitochondria as well as in other compartments. This is even more evident when comparing the quantification of these western blot enrichment profiles (Fig. 2A lower). While ATOM40 (MOM) and cy C (IMS) clearly enrich maximally in the "pure mito" fraction, POMP39 is not enriched from "crude" to "pure mito" and its profile closely resembles the signal of PFR, a flagellar marker. In an alternative fractionation procedure based on digitonin-extraction of whole cells (Schneider et al., 2007b) POMP39-myc is quantitatively recovered in the pellet in which mitochondria and cytoskeletal components are enriched (Fig 2B upper panel).

To further investigate the sub-cellular localization of POMP39 immunofluorescence (IF) was used. The lower panel of Fig 2B shows that the main signal for POMP39-myc appears in the flagellum, with a weaker and diffuse staining of the whole cell body. These results show that another localization of POMP39 seems to be the flagellum. The mitochondrial localization of POMP39 could neither be confirmed nor excluded in this analysis. While flagella are known contaminants of mitochondrial preparations, the purest MOM fraction we prepared was free of flagellar contamination (Niemann et al., 2013). Consequently, the cluster corresponding to the MOM proteome does not contain any known flagellar proteins. In order to further verify that one localization of POMP39 is the MOM, we revisited the mass spectrometry (MS)-based normalized protein abundance profiles that were used to define the MOM proteome of which POMP39 is a component (Niemann et al., 2013). If POMP39 would be a resident flagellar protein that during cell fractionation would associate with the MOM in an artefactual way, other double acylated flagellar proteins should do the same. However, this is not the case as evidenced in Fig. 2C in which the normalized abundance profiles of POMP39 were compared to the one of TbCALp1.3 (Tb927.1.2120), a putative calpain. Many calpains are flagellar proteins (Liu et al., 2010) and TbCALp1.3 has been identified in the flagellar membrane and flagellar matrix proteome of the BSF. Moreover, the protein has the same N-terminal MGCG-sequence as POMP39 and therefore is predicted to be myristoylated and palmitoylated. Finally, TbCALp1.3 was also shown to be palmitoylated by acyl-biotin exchange/streptavidin capture, just as POMP39 (Emmer et al., 2011). As depicted by the black line in the graph of Fig. 2C, POMP39 has a relative abundance maximum in the "pure MOM" fraction, whereas in the

“crude ER” fraction it is approximately 3-fold depleted (Fig. 2C). For TbCALp1.3 however, we find the converse, its maximum abundance is in the “crude ER” fraction and 3-fold less of the protein is found in the “pure MOM” fraction. Noteworthy, a flagellar fraction was not part of this analysis. In summary, although both POMP39 and TbCALp1.3 are dually acylated and found in the flagellum, TbCALp1.3 fractionates very different from POMP39. We therefore conclude that POMP39 is a genuine, dually localized protein that is found in both, the MOM and the flagellum. Another example is an EF-Hand containing putative protein phosphatase. The protein is also dual acylated by myristate and palmitate. In *Leishmania major* the protein was shown to localize to the endomembrane system and the flagellar pocket (FP) by IF (Mills et al., 2007). Consistent with this observed localization, the trypanosome orthologue encoded by Tb927.1.4050 (ca. 60% identity) was in our proteomics study never found in the mitochondrial fraction and had the relative maximal intensity in the crude ER fraction (Niemann et al., 2013). Furthermore, it was not detected in the flagellar proteome (Oberholzer et al., 2011).

3.3 Lack of global myristoylation causes accumulation of POMP39 in the cell body

Next, we were investigating whether the putative acylation(s) influence the localization of POMP39. To that end, immunofluorescence analysis of the cell line that allows for simultaneous down-regulation of TbNMT and expression of POMP39-myc (Fig. 1C) was carried out. The results in Fig. 3 show that in uninduced cells POMP39-myc cannot be detected, as expected serving as a negative control. 24hrs post-induction of TbNMT RNAi the majority of the protein localizes to the flagellum, whereas a fraction is found in the cell body. Finally, the signal in the flagellum diminishes and more of POMP39-myc accumulates in the cell body (Fig 3, left to right panel). The localization of the N-terminally tagged myc-POMP39 and the C-terminally tagged deletion mutant ∆4-POMP39-myc confirmed these results. Both protein variants co-fractionate with soluble cytosolic markers in digitonin extractions (Fig. 4A and B, upper panel). Moreover, immunofluorescence experiments confirm the cytosolic localizations of these proteins to the cell body (Fig. 4A and B, lower panel).

POMP39 is strongly upregulated in the disease-causing BSF of the parasite (Gunasekera et al., 2012; Urbaniak et al., 2012). Consequently, the subcellular localization of POMP39 was also investigated in the BSF. Fig. 4C and D show the results of the digitonin fractionation and the IF analysis of POMP39-myc in BSF trypanosomes. As in the PCF, the C-terminally tagged variant POMP39-myc fractionates with the mitochondrial marker in digitonin extractions (Fig 4C, upper panel) and shows a mainly flagellar localization when analyzed by immunofluorescence (Fig 4C, lower panel). Moreover, as evident form Fig 4D, the localization changes when the tag is placed on the N-terminus and therefore the acylation is abolished: the protein fractionates now with cytosolic markers in digitonin extractions (Fig D, upper panel) and has lost its flagellar localization as visualized by immunofluorescence (Fig 4D, lower panel). In summary, these results show that the presence of the

putative fatty acid(s) is required for proper localization of POMP39 to both the flagella and the MOM.

3.4 POMP39 has a non-essential function.

To investigate the function of POMP39 the protein was subjected to RNAi-mediated ablation in both PCF and BSF trypanosomes. Fig. 5 shows that the growth curves for induced and uninduced cells are virtually identical. Even though the RNAi knockdown is efficient as indicated by Northern blots ablation of the POMP39 mRNAs does not affect growth in neither the PCF (Fig 5A) nor BSF (Fig 5B). Growth at elevated temperature (33°C) in the PCF was also unaffected by the depletion of POMP39. Thus, POMP39 has a non-essential, unknown function under the tested growth conditions or the residual amount of POMP39 that is still present in the induced RNAi cell lines is sufficient to support normal growth.

4. DISCUSSION

POMP39 was identified as a component of the MOM proteome in PCF trypanosomes (Niemann et al., 2013), whereas two earlier proteomic studies of mitochondria and their membranes in PCF *T. brucei* failed to detect the protein (Acestor et al., 2009; Panigrahi et al., 2009). POMP39 was also identified in the flagellar matrix proteome of BSF trypanosomes (Oberholzer et al., 2011), hinting that it might be dually localized. POMP39 contains both a predicted myristoylation and palmitoylation site (99.97% and ≥95% certainty, respectively). The data compiled here suggest that POMP39 is indeed a dual acylated protein, since myristoylation alone does not suffice to confer resistance to carbonate extraction (Peitzsch and McLaughlin, 1993). The myristoylation of POMP39 was interrogated by TbNMT knockdown, which abolishes all myristoylation in *T. brucei*. The TbNMT knockdown resulted in a shift of electrophoretic mobility of POMP39 and a concomitant change of localization, whereas the putative palmitoylation was experimentally addressed in a previous study (Emmer et al., 2011). In line with the global proteomics studies mentioned above (Acestor et al., 2009; Panigrahi et al., 2009; Oberholzer et al., 2011), we show that a fraction of the protein localizes to the flagellum, whereas another fraction is stably associated with the MOM.

A survey of the trypanosomal MOM proteome reveals other candidates that might be acylated. Besides POMP39 there is a single protein, POMP3 (Tb927.9.2670) that is predicted to be dual acylated by myristoylation and palmitoylation. POMP3 has two predicted transmembrane helices and has neither been found in the flagellar nor the mitochondrial proteome before (Acestor et al., 2009; Oberholzer et al., 2011; Panigrahi et al., 2009), but the palmitoylation was experimentally confirmed (Emmer et al., 2011). The function of POMP3 is unknown and the protein is only present in trypanosomatids. However, BLAST searches suggest it may have weak homology to the central domain of the human engulfment and cell motility domain protein 2.

Two proteins of the MOM proteome are predicted to be myristoylated. POMP20 (Tb927.10.9140) is annotated as a hypothetical conserved protein, with high similarity to FAD-dependent oxidoreductases. It has been assigned

to mitochondria before (Panigrahi et al., 2009), but was not identified in the flagellar proteome (Oberholzer et al., 2011). The second protein predicted to be myristoylated is the arginine kinase TbAK1 (Tb927.9.6170). TbAK1 is part of the phosphoarginine energy-buffering system and the C-terminally c-myc tagged protein localizes to the flagellum in both PCF and BSF trypanosomes (Voncken et al., 2013). However, biochemically the protein was not detected in the flagellar proteome (Oberholzer et al., 2011) but in mitochondrial membranes instead (Acestor et al., 2009).

Three proteins of the MOM proteome are palmitoylated (Emmer et al., 2011). These are fatty acyl CoA synthase 4 (ACS4, Tb927.9.4230), a CAAX prenyl protease, (Tb927.11.3120) and POMP12 (Tb927.11.2750) a trypanosomatid-specific protein of unknown function. ACS4 was identified in the mitochondrial membranes (Acestor et al., 2009) and in the flagellar proteome (Oberholzer et al., 2011), while the CAAX prenyl protease was only found to be associated with the proteomes of the mitochondrial membranes (Acestor et al., 2009). POMP12 on the other hand was neither associated with mitochondria nor with flagella in either study (Acestor et al., 2009; Oberholzer et al., 2011; Panigrahi et al., 2009). These results suggest that up to 10% of all MOM proteins are acylated and that some of them, similar to POMP39 investigated in the present study, might be dually localized.

While in trypanosomatids many acylated proteins are at least in part localized in flagella, they are generally found in the endomembrane system in mammalian cells. Mitochondrial localization of acylated proteins is unusual but not without precedent. One example is the NADH ubiquinone oxidoreductase of the mitochondrial inner membrane (Walker et al., 1992). Another example is the myristoylated protein NADH-cytochrome b5 reductase, which in mammalian cells localizes to both, the MOM and the ER (Borgese et al., 1980; Borgese et al., 1986; Borgese and Pietrini, 1980). The protein is inserted post-translationally into the ER and the MOM using a N-terminal anchor and apparently is myristoylated in both of these compartments (Borgese and Longhi, 1990). It was further shown that when the myristoylation site on the N-terminus was abolished by changing the second amino acid from glycine alanine (G2A), the protein is released from the MOM, but remained associated with the ER (Borgese et al., 1996).

How could POMP39 be routed and distributed to both, the MOM and the flagellum? Protein import into mitochondria has been investigated in great detail mainly in yeast. In general, protein import across - or insertion into - the MOM is mediated by a protein complex termed TOM (translocase of the MOM; for recent reviews see e.g. (Becker et al., 2009; Schmidt et al., 2010). Trypanosomes have an unusual TOM complex, the central component of which is ATOM40 (ATOM = archaic translocase of the MOM, see Pusnik et al., 2011; Harsman et al., 2012; Mani et al., 2015). Interestingly, the association of POMP39 with mitochondria remains unaltered upon RNAi-mediated knockdown of ATOM40 (data no shown). Thus, as might be expected for an acylated protein that lacks a membrane-spanning domain, POMP39 localization to the MOM is independent of the protein import machinery. Flagellar targeting of membrane proteins is not very well understood (summarized in e.g. Emmer et al., 2010 and Goldston et al., 2014). However,

as stated above, in trypanosomes many flagellar proteins are acylated (Emmer et al., 2009; Liu et al., 2010), but whether and how the acylation is connected to targeting is not known. A recent hypothetical model suggests that the interplay of NMT, PAT(s) and the presence of a characteristic polybasic region (KKKK) determines, whether dually acylated proteins are targeted to the flagellar or the pellicular membrane (Goldston et al., 2014). The polybasic KKKK stretch, however, is absent from POMP39.

As discussed above POMP39 is likely both, myristoylated and palmitoylated. Palmitoylation is catalyzed by palmitoylacyltransferase (PATs). Twelve PATs and more than 120 target proteins for palmitoylation have been identified in *T. brucei* (Emmer et al., 2009; Emmer et al., 2011). However, so far only one PAT, TbPAT7, could be assigned to a specific substrate. TbPAT7 is non-essential for growth, but required for palmitoylation and subsequent flagellar targeting of calflagin (Emmer et al., 2009). Proteomic profiles of organellar fractions suggest distinct subcellular localization of the trypanosomal PATs. Interestingly, one PAT (TbPAT5) was detected in the highly pure MOM fraction (Niemann et al., 2013). It is thus tempting to speculate that a myristoylated variant POMP39 is palmitoylated by TbPAT5 associated with the MOM, and that this would provide a mechanism for membrane insertion.

Palmitoylation is reversible through the action of acyl protein thioesterase (APT), as was shown in yeast (Duncan and Gilman, 2002). Cycles of acylation/deacylation could thus play an important role in targeting of POMP39 as well. In *T. brucei*, these thioesterases have not been characterized yet. BLAST search of APT1 (*YLR118C*) of *S. cerevisiae* against the *T. brucei* genome reveals as best hit Tb927.8.6390. This protein is annotated as putative lysophospholipase and was found to associate with both, mitochondria and flagella (Acestor et al., 2009; Panigrahi et al., 2009). Our protein abundance profiles also indicate a mitochondrial association (Niemann et al., 2013), priming this enzyme as a potential candidate for the regulation of the mitochondrial localization of POMP39.

In summary our results suggest that the association of POMP39 with the MOM as well as with the flagellar membrane is mediated by acylation. Moreover, a bioinformatics survey suggests that acylation might be a more general way to anchor proteins into the MOM.

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FIGURE LEGENDS

Fig 1. POMP39 is probably myristoylated and palmitoylated. (A) Sketch showing the amino acid sequence of the POMP39 N-terminus and the putative acylation sites (MYR, myristoylation; PALM, palmitoylation). (B) Upper panel, cartoon of the three epitope-tagged variants used in this study. The lower panel shows western blots probed for the c-myc epitope (myc) of whole cell extracts of cell lines expressing these three tagged proteins. (C) Growth curve of the tet-inducible cell line expressing POMP39-myc and allowing simultaneous RNAi-mediated knockdown of TbNMT. Lower panel, western blot of digitonin-solubilized cell extracts of the POMP39-myc/NMT RNAi cell line probed for the c-myc epitope. Cytosolic EF1a serves as a loading control. (D) Carbonate extraction of gradient-purified mitochondria isolated from the POMP39-myc expressing cell line. VDAC, a beta-barrel protein of the MOM and cytochrome C (cy C), a peripheral membrane-associated protein serve as controls.

Fig 2. Dual localization of POMP39 to the MOM and to flagella. (A) Upper panel, western blots of the sub-cellular fractionation of the POMP39-myc cell line to yield gradient-purified mitochondria. 10µg protein were loaded per lane, separated by SDS-PAGE, blotted and probed for the c-myc epitope (myc), the proteins ATOM40 (MOM), cy C (inter membrane space, IMS), cytochrome C1 (cy C1, mitochondrial inner membrane, MIM), mitochondrial heat-shock protein 70 (mHSP70, matrix), the cytosolic marker EF1a and PFR (para flagellar rod, flagellum). The fractions depicted are: whole cells (WC), supernatant 1 (SN1), pellet 1 (P1), crude mitochondria (crude mito) and gradient-purified mitochondria (pure mito) as described (Schneider et al., 2007a). Lower panel, quantified and normalized enrichment profiles of selected markers from the western blots of the upper panel. (B) Upper panel, POMP39-myc expressing cells were subjected to digitonin fractionation. The corresponding western blots were probed for myc, mHSP70 and EF1a. The fractions depicted are whole cells (Tot), supernatant (sup) and pellet (Pe). (B) Lower panel, IF analysis of the POMP39-myc expressing cell line. POMP39-myc is shown in red. 4'6-Diamidin-2-phenylindol (DAPI) in blue stains the nuclear and mitochondrial DNA and ATOM40 shown in green stains the mitochondrial network. Bar: 10µm. (C) Graph showing the MS-based, normalized protein abundance profile for POMP39 (black line) and TbCALp1 (grey line). The fractions used are: microsomal fraction (crude ER), pure mito, crude MIM, crude MOM, pure MOM and a fraction called ER/MOM. The graphs were produced from the data presented in Niemann et al., 2013, which also details the fractionation protocol.

Fig 3. Lack of acylation causes POMP39 to localize to the cell body. (A) IF analysis of the inducible TbNMT RNAi cell line expressing the POMP39-myc stained for POMP39-myc (red), DAPI (blue) and ATOM40 (green). Time of induction of RNAi is indicated. Bar: 10µm.

Fig 4. POMP39 localization likely depends on acylation in both, PCF and BSF *T. brucei*. (A) Digitonin fractionation (upper panel) and IF analysis (lower panel) of the PCF cell line expressing N-terminal tagged POMP39 (myc-POMP39). EF1a serves as cytosolic marker, mHSP0 and dihydrolipoamid-

dehydrogenase (LDH) serve as mitochondrial matrix marker. Bar: 10µm. (B) Digitonin fractionation (upper panel) and IF analysis (lower panel) of the PCF cell line expressing the N-terminal deletion mutant with C-terminal c-myc tag (∆4-POMP39-myc). (C) POMP39-myc expressed in BSF trypanosomes analyzed by digitonin fractionation (upper panel) and IF analysis (lower panel). DIC, differential interference contrast. (D) Digitonin fractionation (upper panel) and IF analysis (lower panel) of myc-POMP39 in BSF trypanosomes.

Fig 5. POMP39 has a non-essential function. Growth curves for RNAi-mediated knockdown of POMP39 in (A) PCF and (B) BSF trypanosomes. Superimposed graphs of induced (grey boxes) and non-induced (black boxes) cells show virtually identical growth. The insets show the corresponding northern blots to verify the absence of the POMP39-mRNA 48hrs post-induction (+tet), the ethidiumbromide (EthBr) stained rRNAs serve as a loading control.

Sup Fig 1. Amino acid sequence of POMP39 orthologues. Alignment of *T. brucei brucei* (Tbb) POMP39 sequences and the *T. brucei gambiense* (Tbg) POMP39 orthologue. Tb927.8.2070 is most similar to the Tbg927.8.1705 sequence. The differences are shown in bold red/bold black.

Sup Fig 2. Nucleotide sequence of POMP39 isoforms. Alignment of *T. brucei brucei* POMP39 sequences (Tb927.8.2070/2260/2270/2280) and the region of Tb927.8.2070 that was used to generate the RNAi construct.

Figure_1

Figure 1 Albisetti et al.

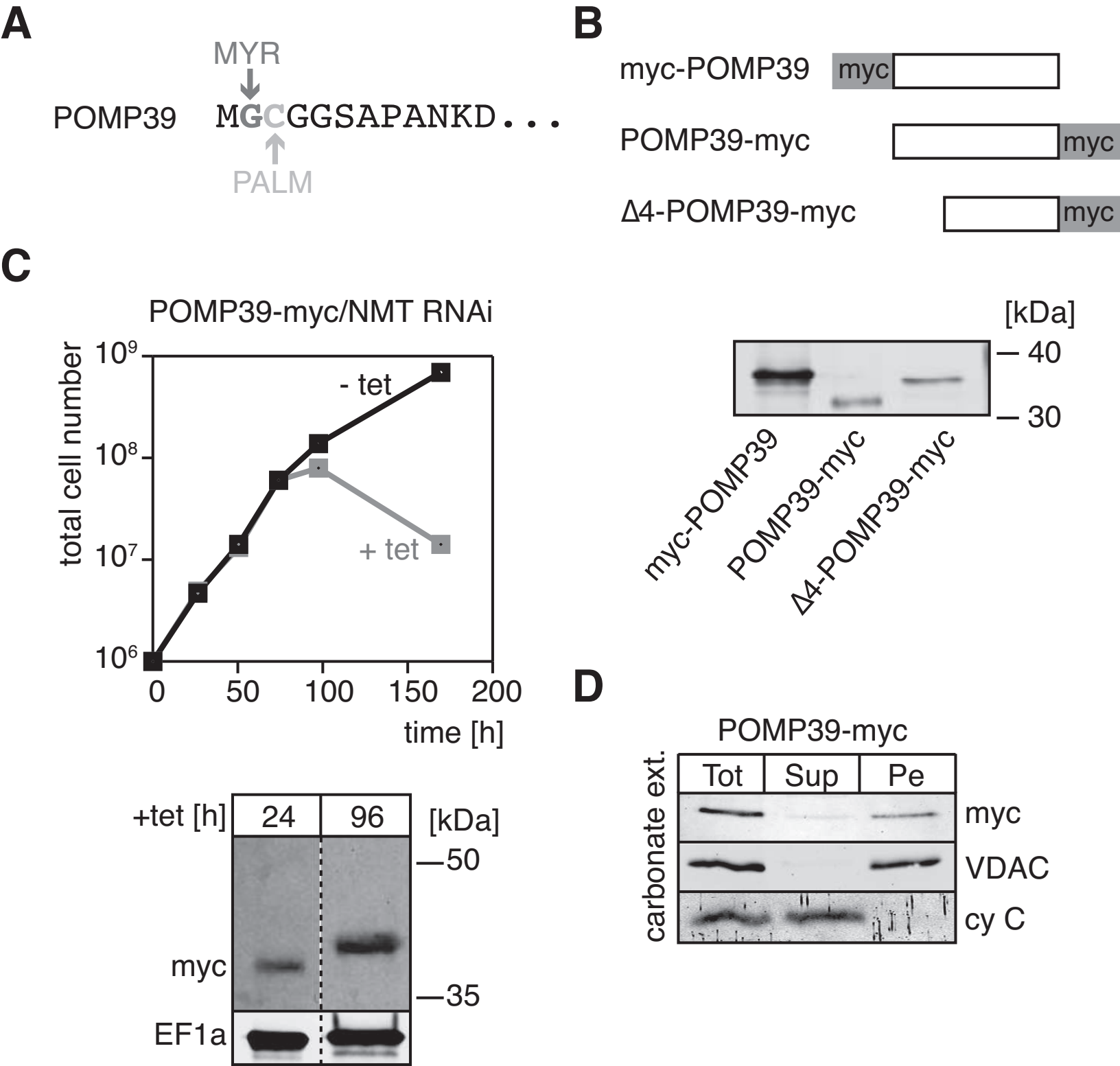


Figure_2

Figure 2 Albisetti et al.

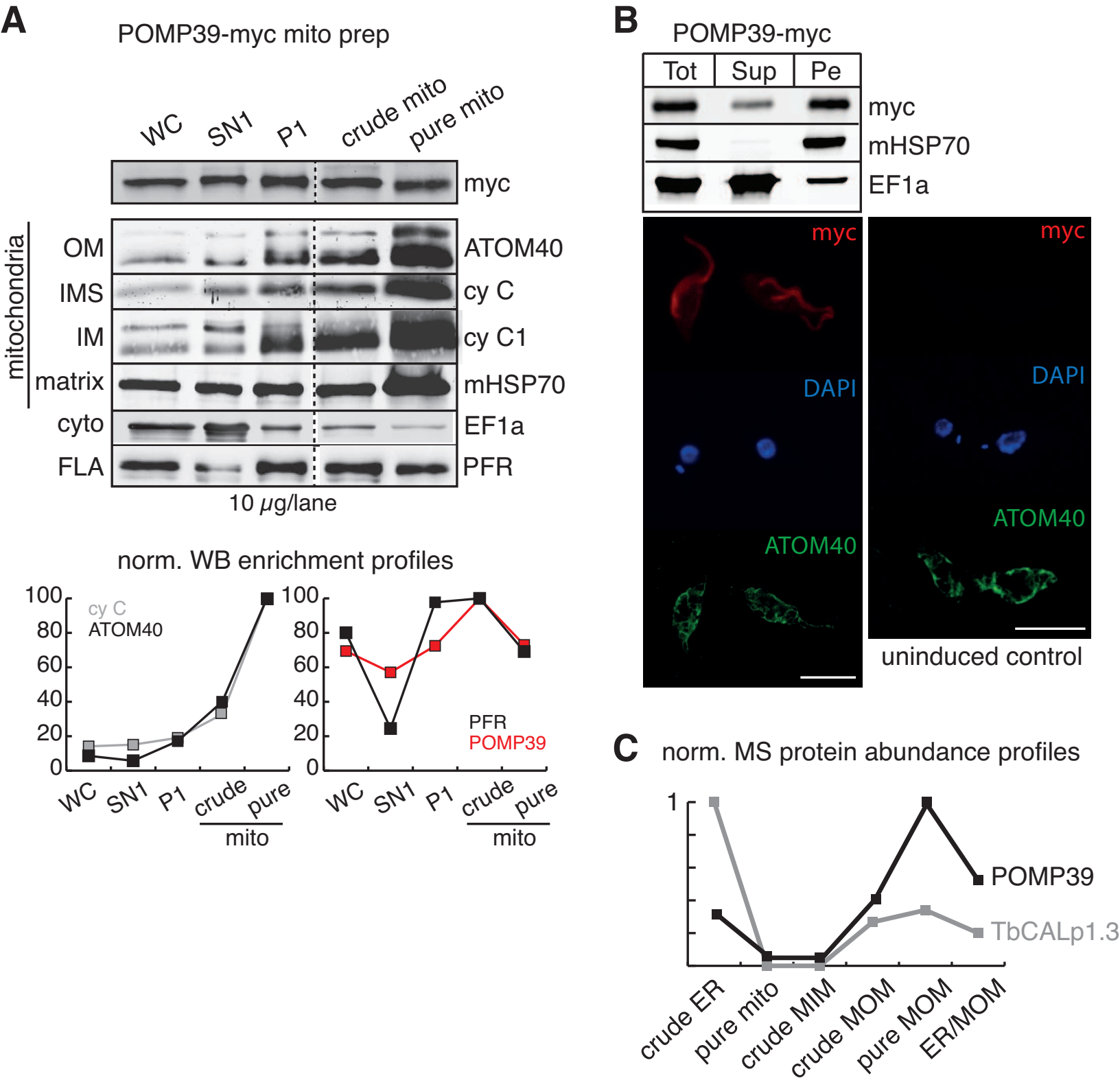


Figure_3

Figure 3 Albisetti et al.

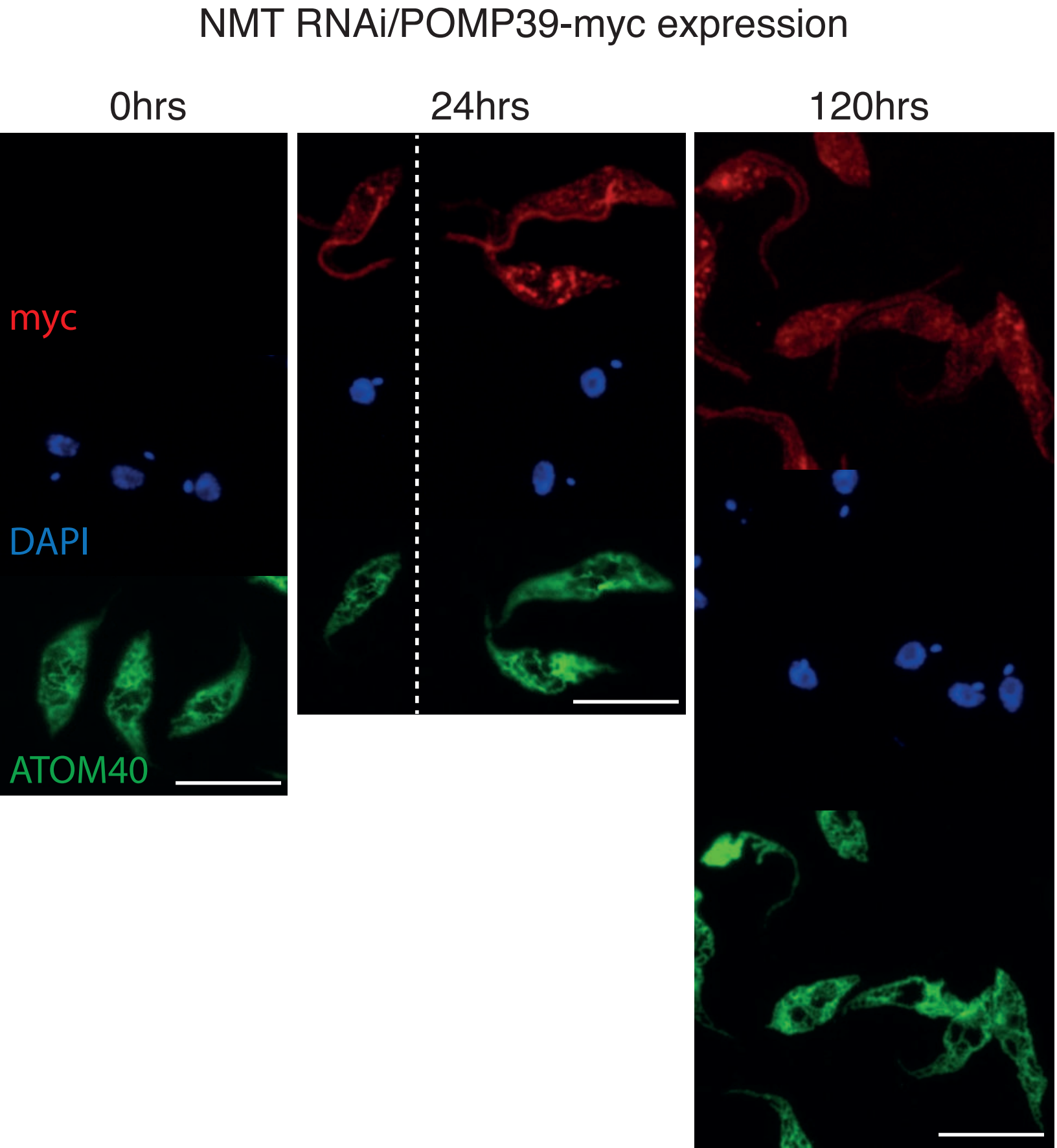


Figure_4

Figure 4 Albisetti et al.

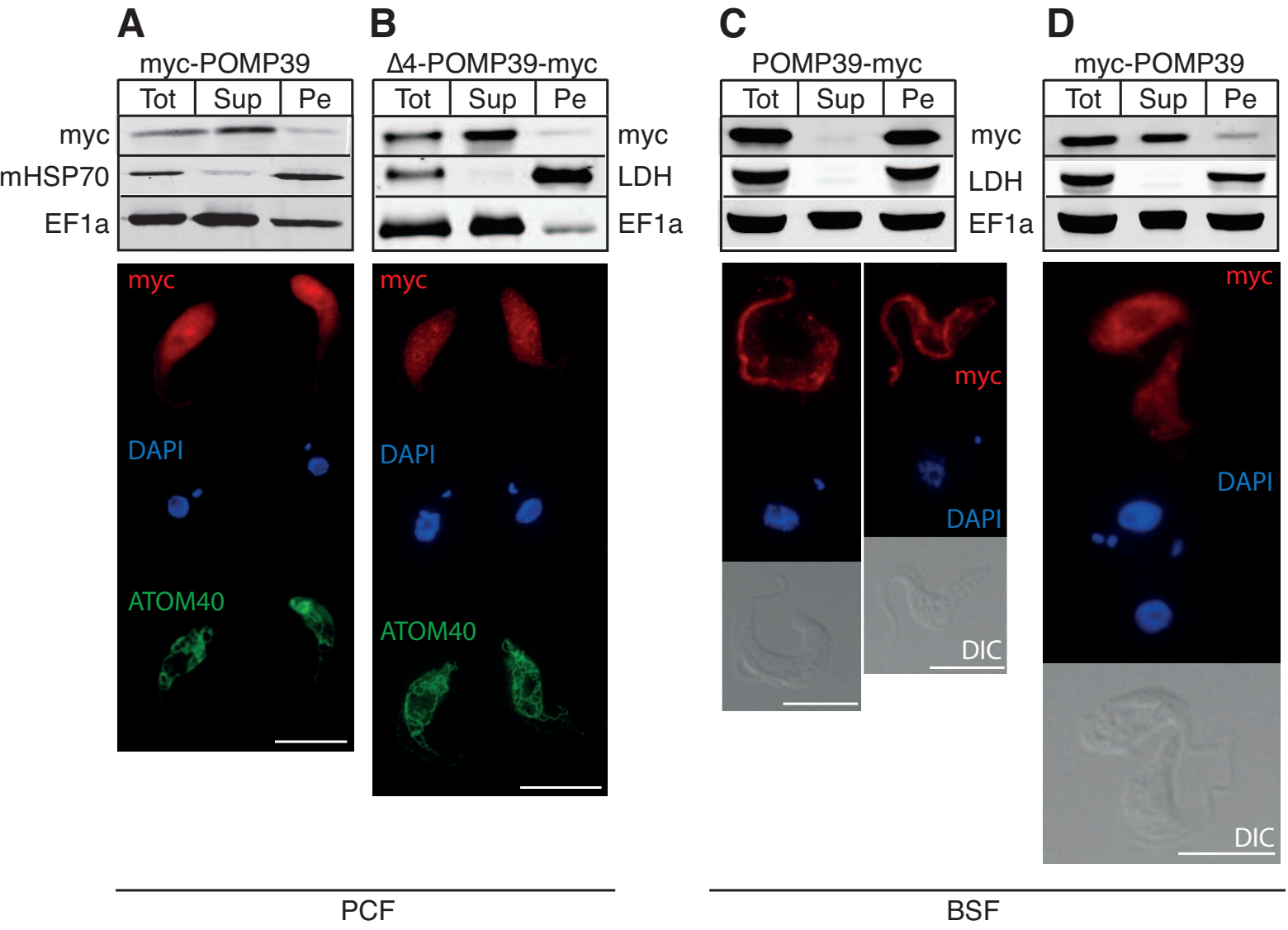
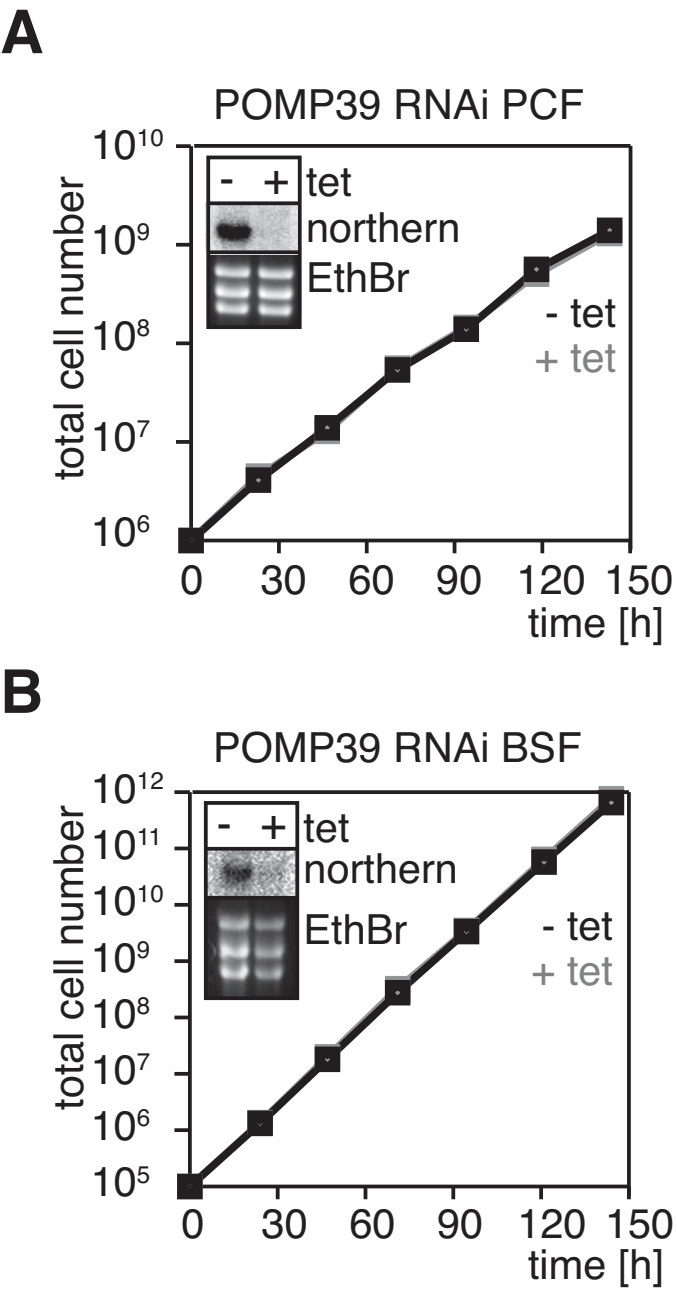


Figure_5 Figure 5 Albisetti et al.



Sup_Figure_1

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Sup_Figure_2

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