

The TOB/SAM complex composition in mitochondria of *Dictyostelium discoideum* during progression from unicellularity to multicellularity*

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Despite its complex life cycle including unicellular and multicellular stages, the slime mold *Dictyostelium discoideum*, a well-known model in biomedical research, has not been used as a model organism in studies on mitochondrial import, including its significance in cellular processes. Moreover, data concerning mitochondrial protein import machinery in *D. discoideum* mitochondria is limited and nothing is known about the impact of that machinery on slime mold life cycle. Here, we focused on the TOB/SAM (topogenesis of the mitochondrial outer membrane β -barrel proteins/sorting and assembly machinery) complex. This complex is localized in the mitochondrial outer membrane and is indispensable for the formation of metabolite exchange and protein import pathways in the membrane, and substantially contributes to the regulation of mitochondrial morphology and distribution. Furthermore, the available data suggests that the TOB/SAM complex variants differ between mitochondria of multicellular and unicellular eukaryotes. Therefore, we decided to determine these variants of the TOB/SAM in mitochondria of *D. discoideum* progressing from single cells to early multicellular stages, when the cells stream together to form a multicellular organism. The results revealed two complex variants of the TOB/SAM complex of about 160 and 600 kDa molecular weight, present in mitochondria of *D. discoideum* cells at the studied stages. The discussed complex variants resemble the ones that have been already detected for the yeast *Saccharomyces cerevisiae*, fungus *Neurospora crassa* and human cells, and one of investigated variants differentiates unicellular and initial multicellular stages of the *D. discoideum* life cycle.

Key words: mitochondria, protein import, TOB/SAM complex, *Dictyostelium discoideum*

Received: 14 March, 2019; **revised:** 17 July, 2019; **accepted:** 16 September, 2019; **available on-line:** 12 November, 2019

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***Acknowledgements of financial support:**

The costs of the article published as a part of the 44th FEBS Congress Kraków 2019 – From molecules to living systems block are financed by the Ministry of Science and Higher Education of the Republic of Poland (Contract 805/P-DUN/2019).

This project was supported by the National Science Centre (Poland) project (grant no. 2012/05/N/NZ3/00293), and the "KNOW RNA Research Centre in Poznań" (grant no. 01/KNOW2/2014).

Abbreviations: MICOS, mitochondrial contact site and cristae-organizing system; TOB/SAM, topogenesis of the mitochondrial outer membrane β -barrel proteins/sorting and assembly machinery; TOM, translocase of the outer membrane; XP_641975.1, Mdm10; XP_642798.1, Tom40; XP_642848.1, metaxin; XP_646058.1, Tob55/Sam50; XP_646782.1, mitofilin/Mic60

INTRODUCTION

The slime mold *Dictyostelium discoideum* is recognized by the National Institute of Health (NIH) as a model of particular value in biomedical research, including mitochondrial diseases (www.nih.gov/science/models). The reason is high homology of *D. discoideum* protein sequences to human proteins (e.g. Eichinger *et al.*, 2005) and its complex life cycle (see Fig. 1). The latter includes different unicellular and multicellular stages which provides a possibility to study cellular processes at intracellular and intercellular levels. This also applies to the mitochondrial protein import process being crucial for the mitochondrial biogenesis and functioning. Most of the mitochondrial proteins are synthesized in the cytosol and therefore have to be imported into various mitochondrial subcompartments.

The mitochondrial outer membrane contains two main import complexes, i.e. the TOM complex (translocase of the outer membrane) and the TOB/SAM complex (topogenesis of the mitochondrial outer membrane β -barrel proteins/sorting and assembly machinery). The TOM complex recognizes, sorts and translocates most of the mitochondrial precursor proteins from the cytosol, whereas the TOB/SAM complex participates in the TOM complex assembly and inserts β -barrel proteins into the outer membrane after translocation of these

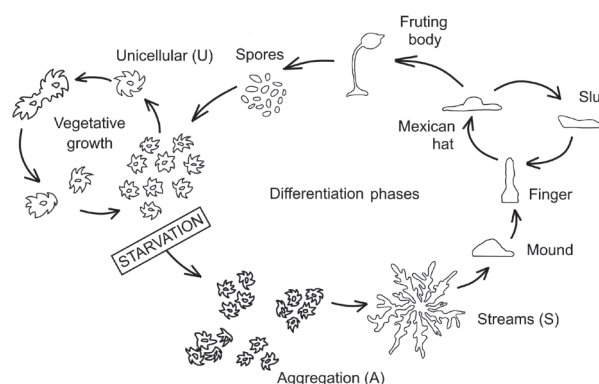


Figure 1. Life cycle of *D. discoideum*.

The vegetative phase (U, unicellular) ceases when food is depleted (bacteria in the soil). Single cells enter differentiation phase, also termed a social phase. After passing the early multicellular stages (A, aggregation; S, streams) cells aggregate and form a motile slug, which ultimately develops into a fruiting body dispersing spores to new feeding sites.

proteins into the intermembrane space by the TOM complex (reviewed by Endo & Yamano, 2010; Neupert, 2015; Höhr *et al.*, 2018; Pfanner *et al.*, 2019). The proteins are exemplified by mitochondrial porin required for metabolite transport, as well as channel forming subunits of the TOM and TOB/SAM complexes, Tom40 and Tob55/Sam50, respectively (Höhr *et al.*, 2018). Thus, the TOB/SAM complex is essential for formation of the metabolite exchange and protein import pathways in the mitochondrial outer membrane and substantially contributes to regulation of mitochondrial functioning.

In studies on the function and interplay of mitochondrial protein import complexes, the yeast *Saccharomyces cerevisiae* is mainly applied as a model organism (e.g. Sokol *et al.*, 2014; Neupert, 2015). The *S. cerevisiae* TOB/SAM complex is proposed to consist of three main subunits, i.e. Tob55/Sam50, Tob38/Sam35 (Tom38), and Tob37/Sam37 (Tom37, Mas37). However, in the case of other eukaryotes, smaller or bigger differences in the complex subunits have been reported. For example, the animal TOB/SAM complex contains metaxin-2 as a counterpart of Tob38/Sam35, as well as metaxin-1 and metaxin-3 as counterparts of Mas37/Sam37 (Sokol *et al.*, 2014), whereas the subunits predicted for the complex of the slime mold *D. discoideum* include Tob55/Sam50 and metaxin as a sole receptor subunit (Buczek *et al.*, 2016; Wojtkowska *et al.*, 2017). It is assumed that the monomeric form of the TOB/SAM complex, composed of the main subunits, has molecular weight of 160–250 kDa, depending on the studied organism (e.g. Kozjak-Pavlovic *et al.*, 2007; Yamano *et al.*, 2010; Ott *et al.*, 2012; Neupert, 2015), although other forms of the complex are also detected due to complex interaction with proteins that are also subunits of other complexes, including the TOM complex (Qiu *et al.*, 2013; Wenz *et al.*, 2015), endoplasmic reticulum-mitochondria encounter structure complex (Korrmann *et al.*, 2009; Yamano *et al.*, 2010; Flinner *et al.*, 2013) and the MICOS (mitochondrial contact site and cristae-organizing system) complex (e.g. Rampelt *et al.*, 2017). The available data indicate that detected forms of the TOB/SAM complex may differ between multicellular organisms, represented by human cells (Kozjak-Pavlovic *et al.*, 2007; Xie *et al.*, 2007; Ott *et al.*, 2012), *Neurospora crassa* (Klein *et al.*, 2012; Lackey *et al.*, 2011) and unicellular ones, e.g. *S. cerevisiae* (Waizenegger *et al.*, 2004; Yamano *et al.*, 2010). This may imply a correlation between the TOB/SAM complex and multicellularity. Here, we present verification of this assumption by analysis of the TOB/SAM complex forms in mitochondria of *D. discoideum* progressing from single cells to early multicellular stages when the cells aggregate and then stream together to form a multicellular organism.

MATERIALS AND METHODS

***Dictyostelium discoideum* growth conditions and the studied life cycle stages.** *D. discoideum* is a social amoeba whose life cycle consists of vegetative growth and a differentiation phase (Flowers *et al.*, 2010; Czarna *et al.*, 2010). The unusual differentiation process starts from aggregation of single cells, which is triggered by nutrient depletion. Then, the developing multicellularity reaches the stage of streams formed by the aggregating cells and followed by a slug formation that culminates as a fruiting body (top of the stalk) consisting of spores (Fig. 1).

The axenic strain AX2 of *D. discoideum* was kindly provided by Prof. Michael Schleicher from Ludwig Maximilian

University of Munich, Germany. *D. discoideum* cells were cultured in axenic growth medium containing 1% w/v proteose peptone, 0.5% w/v yeast extract, 0.45% w/v D-glucose, 0.09% w/v Na₂HPO₄ and 0.23% w/v KH₂PO₄. Cell culture was propagated in 850 ml of the axenic growth medium at 19°C, on a Certomat MO rotary shaker at 90 rpm. Cells were cultured exponentially till the density of approximately 2.0–3.0×10⁶ cells/ml (the doubling time of 10–11 h). These cells constituted the unicellular (U) stage. Cells at aggregation (A) and stream (S) stages were obtained by placing U stage cells into Petri dishes (1.7×10⁶ cells/cm²) with a solid starvation medium called Development Buffer (DB) and containing 5 mM Na₂HPO₄, 5 mM KH₂PO₄, 1 mM CaCl₂, 2 mM MgCl₂, pH 6.5 and 1.25% w/v agar (Fey *et al.* 2007). The cells were harvested after ~23h for A stage, and ~25 h for S stage. The presence of U, A and S stages was confirmed by Nikon Eclipse TE 2000-U microscope. The images were obtained by Nikon DS-1QM camera, and processed by the NIS Elements A 3.10 software.

Immunofluorescent staining. Intact cells were incubated with 200 nM MitoTracker™ Red FM (Invitrogen), a red-fluorescent dye, which stains mitochondria due to accumulation dependent upon the inner membrane potential, for 1 h at 37°C. Cells were then washed in PBS and fixed in 4% paraformaldehyde solution (in PBS) for 20 min at room temperature, seeded on poly L-lysine slides and allowed to dry completely. The cells were subsequently fixed with ice cold methanol and permeabilized with cold acetone for 5 min. Subsequently, the fixed cells were blocked in 5% BSA at room temperature for 1 h and afterwards incubated for 1 h with antibodies raised against Tob55/Sam50 at a dilution of 1:100 in PBS buffer with 5% BSA, at room temperature. Next, the cells were washed in phosphate buffered saline (PBS), and incubated for 1 h at room temperature with goat-anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology) at a dilution of 1:100 (Slocinska *et al.*, 2011). Finally, samples were analyzed under Nikon Eclipse TE 2000-U fluorescence microscope. The images were obtained with a Nikon DS-1QM camera, and processed by the NIS Elements A 3.10 software.

Mitochondria isolation. Cells from U, A and S stages were centrifuged at 600×g for 5 min and then washed twice in phosphate medium A (14.5 mM KH₂PO₄, 5 mM Na₂HPO₄, pH 6) by centrifugation at 600×g for 5 min. Next, the cells were homogenized in medium B (0.38 M sucrose, 20 mM Tris-Cl, 0.5 mM EDTA, 1% defatted bovine serum albumin [BSA], pH 7.5) using glass/Teflon homogenizer and centrifuged at 860×g for 5 min. Supernatant was centrifuged at 10000×g for 15 min. The obtained pellet was suspended in medium C (0.38 M sucrose, 20 mM Tris-Cl, 0.5 mM EDTA, 0.6% defatted BSA, pH 7.2) and centrifuged at 860×g for 5 min to remove remaining cells. Supernatant was centrifuged at 10000×g for 10 min, mitochondrial pellet was suspended in medium D (0.38 M sucrose; 20 mM Tris-Cl, pH 7.2) and centrifuged at 10000×g for 15 min. After this step, the mitochondrial pellet was suspended in medium D and used for further analysis. Efficiency of the mitochondria isolation procedure varied depending on the stage, from 1.5 mg of mitochondrial proteins per 1 g of cells at A and S stages, to 4.5 mg of mitochondrial proteins per 1 g of cells at the U stage. All media used for mitochondria isolation were ice cold and centrifugation procedures were carried out at 4°C.

Protein electrophoresis. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) was performed accord-

ing to Witting and others (Witting *et al.*, 2012). Mitochondria (100 µg) were solubilized in a buffer containing 0.1 M EDTA, 50 mM NaCl, 10% glycerol, 20 mM Tris, 1 mM PMSF, pH 7.4 and 1.25% digitonin (Sigma-Aldrich) for 20 min at 4°C, and centrifuged for 50 min at 14000×g and 4°C. Aliquots of solubilized mitochondria were mixed with Serva Sample Buffer for Blue Native (2× conc.) and loaded on the 6–13% native gradient gel (prepared in 1.5 M ε-amino-n-caproic acid and 0.15 M Bis-Tris, pH 7). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970). SDS-PAGE and BN-PAGE gels were stained with Serva Coomassie Brilliant Blue G-250. To perform Western blot immunodecoration proteins separated by BN-PAGE were transferred to PolyVinylidene Difluoride (PVDF) membrane (Millipore) and by SDS-PAGE to nitrocellulose membrane. After immunodecoration proteins were visualized by the Lumi-Light Western Blotting Substrate (Roche).

Mass spectrometry. Protein bands indicated by Western immunoassays were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) performed in the Mass Spectrometry Laboratory (Institute of Biochemistry and Biophysics, Polish Academy of Sciences Warsaw, Poland). The gel bands were subjected to digestion by a standard procedure where trypsin was used (Promega, Madison, WI, USA). Peptide mixtures were separated by LC-MS/MS prior to molecular mass measurements using LTQ-FTICR on Orbitrap Velos mass. The RP-18 precolumn and nano-HPLC RP-18 column was used for separation of peptides obtained by digestion. Raw data was processed by the Mascot search against the NCBI database (www.ncbi.nlm.nih.gov) restricted to Eukaryota and *D. discoideum* AX2 strain. Peptide mass tolerance was ±20 ppm.

Other methods. Protein concentration was measured by Bradford method, with BSA as a standard (Bradford, 1976). Amino acid sequence of mitofilin/Mic60 was predicted using the blastp algorithm (<https://blast.ncbi.nlm.nih.gov>) and the Pfam model (<http://pfam.xfam.org>).

Antibodies. The antibody against *D. discoideum* Tob55/Sam50 protein, supplied by the Pineda company (Berlin, Germany), was directed against the 20 amino acid N-terminal sequence (NH₂-DDDDIKIKFVNIKSENVFLC-CONH₂). The delivered serum was purified using the modified sulphydrylagarose (Pierce). The first step of purification was binding of the peptide used for immunization with the SulfoLink Coupling Resin. This was done in coupling medium (50 mM Tris, 5 mM EDTA-Na; pH 8.5), using 1 ml of peptide per 1 ml of the resin, for 30 min at room temperature. The coupling efficiency was determined by spectrophotometrical (λ=280nm) estimation of peptide non-coupled fraction concentration. Next, the nonspecific binding sites on the resin were blocked by adding 1 ml of 50 mM cysteine solution to the column at room temperature for 15 minutes with shaking and without mixing for an additional 30 minutes. Afterwards, the column was washed by six resin-bed volumes of 1 M NaCl and stored in the PBS medium. For purification of the antibody against Tob55/Sam50, the column was incubated with 2 ml of serum overnight at 4°C and then washed with PBS. Finally, samples were eluted using 100 mM glycine (pH 3.0) and immediately neutralized by adding 50 µL of neutralization buffer (1 M Tris-Cl, pH 8.8). The elution efficiency was estimated spectrophotometrically (λ=280nm). Samples of interest were desalted in the presence of PBS and stored

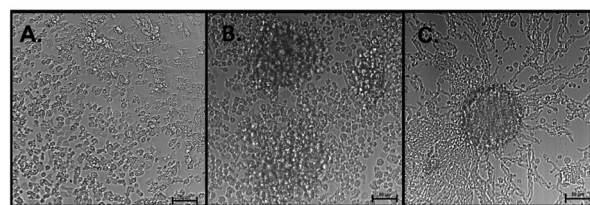


Figure 2. The early multicellular stages of the *D. discoideum* life cycle.

Samples were analyzed with a Nikon Eclipse TE 2000-U light microscope. The images were obtained with a Nikon DS-1QM camera. U, unicellular stage; A, aggregation stage; S, streams.

at –20°C. The antibody against human metaxin-1 protein was purchased from Sigma-Aldrich.

RESULTS

The early multicellular stages of the *D. discoideum* life cycle

To monitor changes of the TOB/SAM complex variants during transition from unicellularity to multicellularity, three stages: unicellular (U), aggregation (A) and streams (S) were selected. The occurrence of those stages in the *D. discoideum* life cycle is depicted in Fig. 1 and was confirmed by light microscopy as shown in Fig. 2. The starvation of U stage cells resulted in the A stage (Fig. 2B), characterized by the presence of irregular clusters of various amounts of cells. Further starvation of cells has led to the S stage (Fig. 2C), formed by cells in an aggregation process, and followed by mound formation (Fig. 1). As shown in Fig. 2C, cells at the S stage gathered by migrating in organized streams, finally producing a structure similar to a star or a flower.

Detection of the TOB/SAM complex main subunits in mitochondria of unicellular and early multicellular stages

As we had previously predicted the main subunits of the *D. discoideum* TOB/SAM complex using available genomic and transcriptomic sequences (Wojtkowska *et al.*, 2012; Buczek *et al.*, 2016), we decided to confirm the presence of the Tob55/Sam50 and metaxin subunits at a protein level. The designed polyclonal antibody against Tob55/Sam50 (Materials and Methods) was used to confirm the presence of Tob55/Sam50 in mitochondria of intact cells at the studied stages (Fig. 3A), as well as in mitochondria isolated from these cells (Fig. 3B). As shown in Fig. 3A, staining of mitochondria with MitoTracker™ Red FM (Fig. 3A.1) and subsequent incubation with the antibody against Tob55/Sam50 (Fig. 3A.2) co-localized in the merged image (Fig. 3A.3), indicating the presence of Tob55/Sam50. The protein was also detected in separated mitochondrial proteins by Western immunoassays (band ca. 45 kDa; Fig. 3B). Moreover, signals obtained with the antibody against Tob55/Sam50 for mitochondria isolated from cells at the studied stages were confirmed by LC-MS/MS using the corresponding band in SDS-PAGE gels (Table S1 at <https://ojs.ptbioch.edu.pl/>). The presence of metaxin was also confirmed in mitochondria isolated from cells at the studied stages by application of a commercially available human metaxin-1 antibody (Fig. 3B), as well as by LC-MS/MS using the corresponding band in SDS-PAGE gels (Table

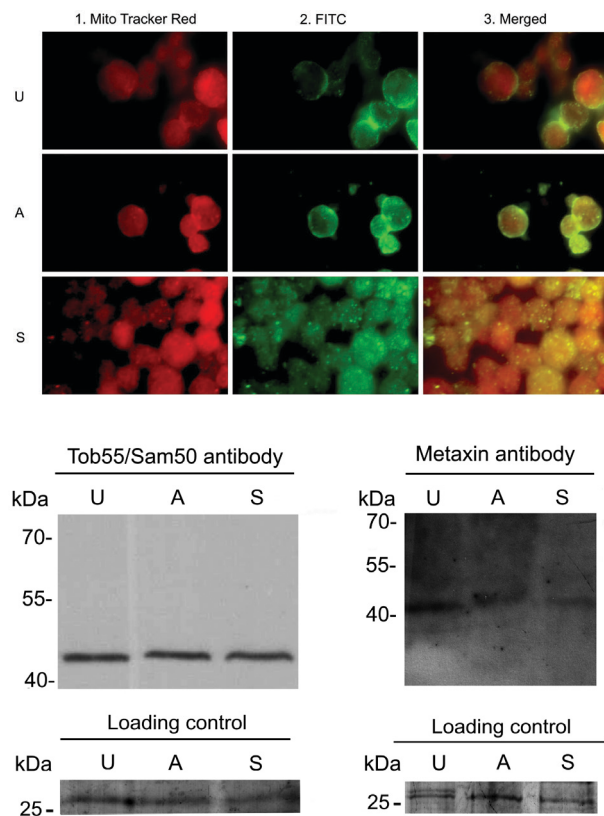


Figure 3. Detection of the *D. discoideum* TOB/SAM complex main subunits at a protein level.

(A) Immunofluorescence localization of Tob55/Sam50. The localization was performed for single cells (U), as well as cells from the early developmental stages (U, A and S). **Panel 1**, staining with MitoTracker™ Red FM (Invitrogen) to visualize mitochondria; **Panel 2**, incubation with goat anti-rabbit IgG recognizing the antibody against Tob55/Sam50 protein and conjugated to fluorescein isothiocyanate (FITC); **Panel 3**, the merged image. Specimens were analyzed with a Nikon Eclipse TE 2000-U fluorescence microscope. Images were obtained with a Nikon DS-1QM camera. (B) Immunodetection of Tob55/Sam50 and metaxin among mitochondrial proteins isolated from the cells described in (A), separated by 12% SDS-PAGE and immunodecorated by respective antibodies after Western blotting. The results shown in (A) and (B) are typical for 3 independent experiments. Loading control – a 25 kDa protein band shown on part of a Coomassie stained gel.

S1 <https://ojs.ptbioch.edu.pl/>). The molecular weight of this protein was estimated to be at about 46 kDa.

Estimation of the TOB/SAM complex variants in mitochondria of unicellular and early multicellular stages

The application of western immunoassay on BN blots with antibodies against Tob55/Sam50 and human

Table 1. Components of the TOB/SAM complex variants from the U, A and S developmental stages in *D. discoideum* mitochondria

app. molecular weight	Subunits detected by	
	immunodetection	LC-MS/MS
600 kDa	Tob55/Sam50; metaxin	Tom40; mitofilin
160 kDa	Tob55/Sam50	Mdm10; mitofilin

metaxin-1 to detect possible variants of the TOB/SAM complex in mitochondria isolated from cells at U, A and S stages, indicated the presence of two distinct forms of the complex independently of the studied stage (Table 1). Their molecular weights were estimated to be at about 600 and 160 kDa. Importantly, metaxin was detected in the larger complex only. Other putative partners or subunits of the TOB/SAM complex variants could not be detected by immunoassays due to the lack of specific antibodies. Therefore, we applied LC-MS/MS to identify them. As a result, additional proteins were identified. In the 600 kDa complex, Tom40 (Wojtkowska *et al.*, 2012) and mitofilin/Mic60 were found (Table 2), the latter for the first time in this study (Table S2 at <https://ojs.ptbioch.edu.pl/>). Importantly, mitofilin/Mic60 was detected only at the U stage. A putative nucleotide sequence of mitofilin/Mic60 was found by blastp and Pfam model (Fig. S1 at <https://ojs.ptbioch.edu.pl/>), and used to detect the mitofilin domain (Yang *et al.*, 2012; Ott *et al.*, 2015) in a putative sequence obtained by LC-MS/MS. Interestingly, in the 160 kDa complex, we detected Mdm10, but not the Tom40 protein (Table 1). Notably, Mdm10 had been previously predicted in *D. discoideum* by bioinformatics analysis (Buczek *et al.*, 2016) but at a protein level it was confirmed for the first time in this study (Table S2 at <https://ojs.ptbioch.edu.pl/>).

DISCUSSION

The available data on different variants of the TOB/SAM complex detected for unicellular (*S. cerevisiae*) and multicellular (*N. crassa* and human cells) organisms suggests a correlation between this complex and the level of organism organization (Table 3). *D. discoideum* cells progressing from the single cell (U) stage to early multicellular (A and S) stages, when single cells aggregate and then stream together forming a multicellular structure, constitute a useful model to study a shift between unicellularity and multicellularity. However, the assumed relationship is not confirmed by the obtained results as they indicate that the TOB/SAM complex variants do not differ between the stages. Namely, the same two variants of the complex of molecular weight of about 600 and 160 kDa are present in mitochondria isolated from cells at these stages (Fig. 4).

Table 2. The TOB/SAM complex subunits identified at the protein level in mitochondria of *D. discoideum* cells progressing from unicellularity to early multicellularity

Identified protein	Accession number	Proteins identified at	
		the genome/transcriptome level	the protein level
Tob55/Sam50	XP_646058.1	Wojtkowska <i>et al.</i> , 2012	this study
Tom40	XP_642798.1	Wojtkowska <i>et al.</i> , 2012	Wojtkowska <i>et al.</i> , 2015
metaxin	XP_642848.1	Buczek <i>et al.</i> , 2016	this study
Mdm10	XP_641975.1	Buczek <i>et al.</i> , 2016	this study
mitofilin/Mic60	XP_646782.1	this study	this study

Table 3. Variants of the TOB/SAM complex of *S. cerevisiae*, *N. crassa*, human and *D. discoideum* mitochondria

<i>S. cerevisiae</i> (Qiu <i>et al.</i> , 2013)	<i>N. crassa</i> (Klein <i>et al.</i> , 2012)	Human (Ott <i>et al.</i> , 2012)	<i>D. discoideum</i> this study
650 kDa supercomplex TOB/SAM: Tob55/Sam50; Sam37, Sam35; TOM: Tom40, Tom22, Tom20, Tom70, Tom6, Tom7, Tom5	> 600 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35	> 700 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35; mitofilin	> 650kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35;
440 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35; Mdm10	200 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35; Mdm10	250 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35	Tom40; mitofilin*
200 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35	140 kDa TOB/SAM: Tob55/Sam50; Sam37, Sam35		Tob55/Sam50; Mdm10; mitofilin*

The detected variants of the *D. discoideum* TOB/SAM complex correspond to the monomeric variant of the complex composed of the main subunits (160 kDa) and the form interacting with other proteins (600 kDa). A monomeric variant of comparable size has been reported for *S. cerevisiae* (Waizenegger *et al.*, 2004; Yamano *et al.*, 2010; Qiu *et al.*, 2013), *N. crassa* (Lackey *et al.*, 2011; Klein *et al.*, 2012) and human cells (Kozjak-Pavlovic *et al.*, 2007; Ott *et al.*, 2012) (Table 3). Importantly, the variant of the *D. discoideum* complex does not contain metaxin. As the protein is detected by anti-metaxin-1 antibody (Fig. 3), it might be assumed that this form is missing a counterpart of Tob37/Sam37 (Sokol *et al.*, 2014), although may contain subunits still not detected. Tob37/Sam37 has been shown to be not essential and to act at the late stages of β -barrel assembly by assisting protein release from the complex (Chan & Lithgow, 2008; Dukanovic *et al.*, 2009). Accordingly, in *S. cerevisiae* and *N. crassa*, mitochondria variants missing the Tob37/Sam37 protein have been also detected (Waizenegger *et*

al., 2004; Lackey *et al.*, 2011). Furthermore, it has been reported that in the human mitochondria, Tob55/Sam50 and metaxins seemed to be present in different complexes (Kozjak-Pavlovic *et al.*, 2007). On the other hand, the *D. discoideum* form contains Mdm10 which is not present in the human mitochondria but is also detected in the forms of the TOB/SAM complex present in *N. crassa* (Lackey *et al.*, 2011; Klein *et al.*, 2012) and *S. cerevisiae* (Waizenegger *et al.*, 2004; Yamano *et al.*, 2010; Qiu *et al.*, 2013) mitochondria, although data concerning the size of the resulting complexes are not consistent.

The 600 kDa form of the TOB/SAM complex corresponds to the 600–700 kDa supercomplex detected for *S. cerevisiae* (Qiu *et al.*, 2013; Wenz *et al.*, 2015) and human cells (Kozjak-Pavlovic *et al.*, 2007; Ott *et al.*, 2012). In the case of *S. cerevisiae* mitochondria, the 650–700 kDa supercomplex has been shown to contain the TOM complex interacting with the TOB/SAM complex and to promote substrate channeling in the β -barrel pathway (Qiu *et al.*, 2013; Wenz *et al.*, 2015) (Table 3). Thus, the presence of Tom40 in the 600 kDa *D. discoideum* form could be considered in two aspects. Firstly, Tom40 is the most abundant subunit of the TOM complex (Sirrenberg *et al.*, 1997). As the molecular weight of the *D. discoideum* TOM complex has been estimated to be about 430 kDa (Wojtkowska *et al.*, 2015), it can be assumed that the 600 kDa form may represent the supercomplex consisting of the TOB/SAM (160 kDa) and the TOM complex. Secondly, Tom40 being the β -barrel protein itself is imported by the TOB/SAM complex. Thus, its presence in the form of the TOB/SAM complex may also reflect its import process. However, Tom40 has not been detected in the 600–700 kDa complexes in human mitochondria.

The presence of mitofilin/Mic60, a key component of the MICOS complex (Harner *et al.*, 2011; Malsburg *et al.*, 2011; Eydt *et al.*, 2017), in the 600 kDa form of the *D. discoideum* TOB/SAM complex at the U stage, appears to confirm the interaction between the protein and the main subunits of the TOB/SAM complex. This interaction has been shown for *S. cerevisiae* and human mitochondria (e.g. Ott *et al.*, 2012; Rampelt *et al.*, 2017) and is regarded to be mediated by Tob55/Sam50 (Ott *et al.*, 2012; Xie *et al.*, 2007; Ding *et al.*, 2015) and/or metaxin (Xie *et al.*, 2007). Accordingly, the yeast homologue of mitofilin/Mic60, termed Fcj1, has been shown to be important for association of the TOB/SAM complex with mitochondrial contact sites, resulting in stabilization of crista junctions in close proximity to the outer membrane (Korner *et al.*, 2012). In addition, the TOB/SAM complex is also described as an anchor for the MICOS complex subunits (Harner *et al.*, 2011). Moreover, the presence of mitofilin/Mic60 in the 600 kDa vari-

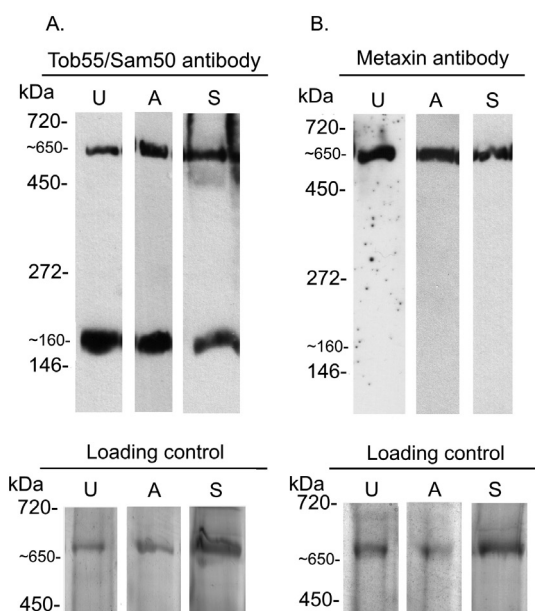


Figure 4. Estimation of the *D. discoideum* TOB/SAM complex forms in mitochondria of single cells, as well as cells from the early multicellular stages.

Mitochondria solubilized in the presence of 1.25% digitonin were separated by BN-PAGE and the obtained Western blots were immunodecorated by Tob55/Sam50 and metaxin antibody. The data presented are typical for 3 independent experiments. (U) unicellular; (A) aggregation; (S) streams. Loading control – a 650 kDa complex band shown on part of a Coomassie stained BN gel.

ant of the *D. discoideum* TOB/SAM complex, may result from the protein interaction with the TOM complex, as proposed for *S. cerevisiae* (Qiu *et al.*, 2013; Rampelt *et al.*, 2017), but not observed for human mitochondria. Thus, it can be assumed that the interaction of the TOB/SAM complex with the MICOS complex may occur at the U stage mitochondria and may include the TOB/SAM complex interaction with the TOM complex.

In summary, the detected forms of the *D. discoideum* TOB/SAM complex, i.e. the 160 and 600 kDa forms, appear to be similar to the forms found in *S. cerevisiae*, *N. crassa* and human mitochondria. The 600 kDa form seems to differentiate between unicellular and early multicellular stages by putative interaction with the MICOS complex subunit, although the next stages of the *D. discoideum* life cycle should be studied to observe more spectacular differences in the TOB/SAM complex forms.

Other acknowledgement

We would like to thank Arkadiusz Urbański, Ph.D. from the Department of Animal Physiology and Development, Faculty of Biology, AMU Poznań for technical support in preparing *D. discoideum* photos from light microscope. The equipment used was sponsored in part by the Centre for Preclinical Research and Technology (CePT), a project co-sponsored by European Regional Development Fund and Innovative Economy, The National Cohesion Strategy of Poland.

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