

## SUPPLEMENTAL MATERIAL

**Figure S1. *Gi*Tom40 biotinylation by cytosolic BirA.** *Gi*Tom40 is biotinylated when co-expressed with cytBirA. BAP-tagged *Gi*Tom40 was detected by streptavidin conjugated with Alexa 488 (green). Cytosolic BirA was detected with anti-HA tag antibody (red). Nuclei were stained with DAPI (blue).

**Figure S2. Localization of N-terminally truncated *Gi*MOMP35 and full length GL50803\_9296.**

(A) When *Gi*MOMP35 was expressed without two predicted N-terminal transmembrane domains ( $\Delta$ *NGi*MOMP35) its mitochondrial localization was abolished and the protein was localized in the cytosol.  $\Delta$ *NGi*MOMP35 was detected by anti-HA tag antibody (green) and mitochondria were labeled by anti-GL50803\_9296 antibody (red), nuclei were stained with DAPI (blue). (B) During the protease protection assay the HSP from wild type *G. intestinalis* was solubilized in isotonic, hypotonic, NaCl-containing or Triton-containing buffer. The suspensions were treated either by pipetting or sonication and subjected to the treatment by proteinase K. The resulting samples were resolved on SDS-PAGE, transferred to membrane and probed for the matrix marker protein IscS, outer membrane protein Tom40 and GL50803\_9296. While Tom40 was sensitive to the protease in the isotonic buffer, both GL50803\_9296 and IscS remained protected by mitochondrial membranes. These data suggest the matrix localization of GL50803\_9296.

**Figure S3. The compiled lists of the most significant hits obtained from the purification**

**experiments of five mitochondrial proteins.** The results were divided into two sets derived either from the purification of the matrix or the outer membrane proteins. The results were colour-coded in (A): the proteins found in *Gi*Pam18- and *Gi*Tim44-specific datasets are shown in green, the proteins found in *Gi*Pam18- and *Gi*Hsp70-specific datasets are shown in red, the proteins found in *Gi*Tim44- and *Gi*Hsp70-specific datasets are shown in blue and the proteins common to all three datasets are shown in yellow. In (B), the proteins found in *Gi*Tom40- and *Gi*MOMP35-specific datasets are shown in violet. The novel mitochondrial proteins confirmed by episomal expression are shown with red

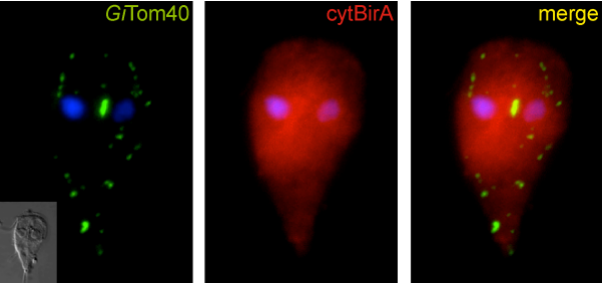
borders and the proteins whose episomal expression resulted in a different than mitochondrial localization are shown in grey. The proteins, the episomal expression of which induced a lethal phenotype, are labelled by a black cross.

**Table S1. Primers used in the study.**

**Table S2. The list of genes episomally expressed in *G. intestinalis*.**

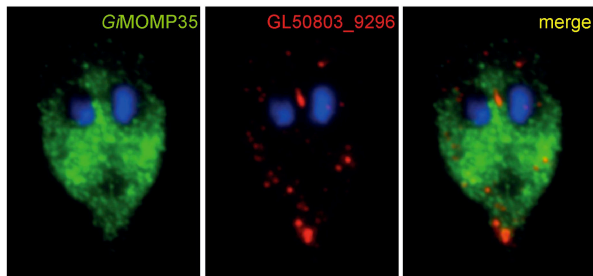
**Table S3. Distribution of the identified proteins in metamonads.**

The genome project of *T. vaginalis* and *S. salmonicida* were searched for the presence of the novel *G. intestinalis* mitochondrial proteins.

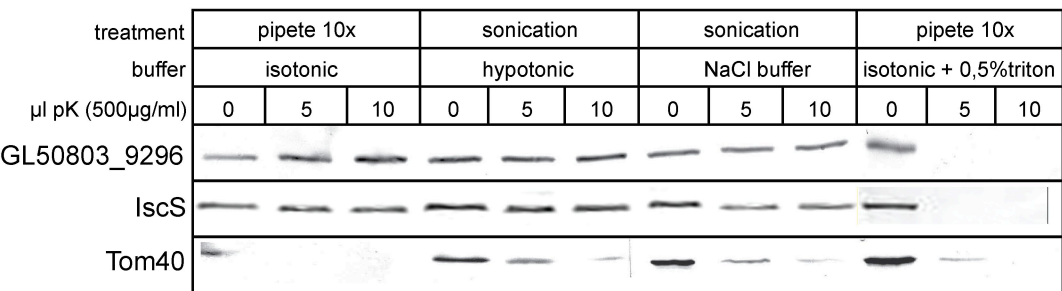


Supplementary Figure 1

A



B



A

## Mitosomal matrix

<i>GiPam18</i>	<i>GiTim44</i>	<i>GiMthHsp70</i>
Pam18	Tim44	Hsp70
Hsp70	glutaredoxin	GL50803_16424
glutaredoxin	Hsp70	GL50803_12229
Mge1	GPP	GL50803_16891
IscA	Mge1	GL50803_8148
Cpn60	GL50803_16424	GiOR-1
GPP	IscA	Tim44
Tim44	Ferredoxin	GL50803_7035
Nfu	GL50803_137745	GL50803_12598
IscU	GL50803_137712	GL50803_14818
GL50803_112784	GL50803_16386	IscA
GL50803_31998	GL50803_92741†	GL50803_16350
GL50803_16424	GL50803_3491	GL50803_16985
GL50803_17276	GL50803_15023	GL50803_17312
GL50803_9296	GL50803_113722	Ferredoxin
GL50803_16978	GL50803_17276	GL50803_3141
GL50803_7608	IscS	GL50803_3957
GL50803_16049	Cpn60	GL50803_8726
GL50803_15991	GL50803_13555	GL50803_8955
GL50803_6544	Pam18	GL50803_9148
GL50803_15487	GL50803_14216	GL50803_14198
GL50803_6317	GL50803_17492	GL50803_16926
GiOR-1	GL50803_24842	GL50803_2822
GL50803_27910	GiOR-1	GL50803_4415
GL50803_137726	GL50803_2305	GL50803_8974
GL50803_15476	GL50803_8358	GL50803_8243
GL50803_9363	GL50803_40390	IscU
GL50803_92741†	GL50803_2527	GL50803_17258
GL50803_8358	GL50803_16944	GL50803_2338
GL50803_23630	GL50803_5305	GL50803_4692

B

## Outer mitosomal membrane

<i>GiTom40</i>	<i>GiMOMP35</i>
Tom40	MOMP35
GL50803_13922	GL50803_14818†
GL50803_10971	GL50803_16926
GL50803_114210	GL50803_16985
GL50803_7188	GL50803_14198
MOMP35	GL50803_10971
GL50803_8152	GL50803_29796
GL50803_14297	GL50803_16981
GL50803_16424	GL50803_8623
GL50803_22291	GL50803_8460
GL50803_91712	GL50803_16424
GL50803_137685	Tom40
GL50803_17276	GL50803_2822
GL50803_96818	GL50803_3957
GL50803_14469	GL50803_4590
GL50803_33674	GL50803_12224
GL50803_6939	GL50803_8955
GL50803_22587	GL50803_7188
GL50803_31295	GL50803_7778
GL50803_7242	Hsp70
GL50803_21505	GL50803_10452
GL50803_40224	GL50803_4852
GL50803_9529	GL50803_12229
GL50803_15104	GL50803_17312
GL50803_16679	GL50803_22291
GL50803_16957	GL50803_6007
GL50803_8471	GL50803_17484
GL50803_86468	GL50803_22587
GL50803_4852	GL50803_7348
GL50803_12945	GL50803_13922

**Table S1. Primers used in the study**

BirA MluI F	GATCACGCGTATGAAGGATAACACCG
BirA NsiI R	CATGATGCATTTTTCTGCACTACGC
GiMgel-BirA NdeI F	ATGCCATATGGCGCTTTCTGCAC
GiMgel-BirA MluI R	CATGACGCGTGATCTCGTGAGATGT
GiPam18-BAP NdeI F	CTAGCATATGCTACGTGTACTGAGCG
GiPam18-BAP BglII, BamHI R	CATGAGATCTTACTCGTGCCATTCTATCTTCTGA GCCTCAAAGATGTCATTTAGGCCGGATCCGCATC TGTTCTGAGCTTC
GiTim44-BAP AseI F	ACTGATTAATATGAAAAGTTTTACGCCCTAT
GiTim44-BAP BamHI R	CATGGGATCCATAGAAATACGTCCGGCTTTGC
GiMtHsp70-BAP NdeI F	CATGCATATGCTTAAGCGGCACGTAAG
GiMtHsp70-BAP BamHI R	CATGGGATCCCGTGCTAGTGGCGCGTC
GiTom40-BAP AseI F	CATGATTAATATGCCCTTTCCTGGTCT
GiTom40-BAP BglII R	CATGAGATCTTTGTGATTGCTCGAAG
GiMOMP35-BAP NdeI F	CATGCATATGCCGGCTCACAGACACAG
GiMOMP35-BAP BglII R	CATGAGATCTATTTAGCCTTGCTTGG
GL50803_14845 AseI F	CATGATTAATATGAAAAGTTTTACGCCCTA
GL50803_14845 PstI	CATGCTGCAGATAGAAATACGTCCGC
DHFR MluI F	CATGACGCGTATGGTTCGACCATTGAAC
DHFR PstI R	CATGCTGCAGGTCTTTCTTCTCGTAGAC
GiMgel-DHFR NdeI F	ATGCCATATGGCGCTTTCTGCAC
GiMgel-DHFR MluI R	CATGACGCGTG TAGATATCATTATCAC
GL50803_14818 NdeI F	CATGCATATGCCACCTCTGGTTCCC
GL50803_14818 NsiI R	CATGATGCATACTCAGGAGCCATTTCATC
GL50803_12229 NdeI F	CATGCATATGCGGTGCCAGGATGT
GL50803_12229 NsiI R	CATGATGCATGTTATAGAAGTATGTATT
GL50803_16891 AseI F	CATGATTAATATGAGTACTTTGACTCGT
GL50803_16891 NsiI R	CATGATGCATCCGTTTACTCAGCATGGC
GL50803_7035 NdeI F	CATGCATATGATGTACAATTTGATGCTT
GL50803_7035 PstI R	CATGCTGCAGATCTGACGAGGATGAGGT
GL50803_8358 NdeI F	CATGCATATGAAGTACATTTTTGAT
GL50803_8358 PstI R	CATGCTGCAGGTTGAACTTCTTGGGCTC
GL50803_16386 NdeI F	CATGCATATGATGCGTCCAACCAGGACC
GL50803_16386 NsiI R	CATGATGCATGTGTGAAGATGACTCGAC
GL50803_10971 AseI F	CATGATTAATATGAATGTTGCTGTCTTT
GL50803_10971 SbfI R	CATGCCTGCAGGGTATTCAGCGAAAAGCCA
GL50803_22291 NdeI F	CATGCATATGGCCGGAAGAAGGAG
GL50803_22291 PstI R	CATGCTGCAGCACACTGTCTGTGCTCCC
GL50803_4852 AseI F	CATGATTAATATGGAGGACCTTCCCTTC
GL50803_4852 NsiI R	CATGATGCATTA CTATCGAGAAACGACG
GL50803_16424 AseI F	CTAGATTAATCCCAGTAGTAAATAC
GL50803_16424 NsiI R	CTAGATGCATGTAGCGACGATTACCGGATGA

GL50803_8148 NdeI F	CATGCATATGATGAGGCCTCGTCCT
GL50803_8148 PstI R	CATGCTGCAGGTACGTTCTATAGAC
GL50803_12598 NdeI F	CATGCATATGATGGAAGACGAGATG
GL50803_12598 NsiI	CATGATGCATAAGGCCATCACTAGG
GL50803_17276 NdeI	CATGCATATGATGCCTCGGCGCTTC
GL50803_17276 SbfI R	CATGCCTGCAGGAGACGGTCTTGAAGCATT
GL50803_27910 NdeI F	CGATCATATGCTTGCGAGCTTGCCACAGAC
GL50803_27910 PstI R	CGATCTGCAGCCGAGGAGAATCTGGTGGCA
GL50803_3491 NdeI F	CGATCATATGCTATCTTTGACTACCATCAAG
GL50803_3491 PstI R	CGATCTGCAGCTCGTTCGCACTGGCGTGTG
GL50803_92741 NdeI F	CGATCATATGGCACTGTCTTCACGCCAAA
GL50803_92741 PstI R	CGATCTGCAGATTTATCCCGAAAGCAATAT
GL50803_13922 XhoI F	CTAGCTCGAGATGTTACTTCTCCTCTGCTGG
GL50803_13922 SbfI R	CATGCCTGCAGGTTACGCTGCCATGAGAGATAT
GL50803_7188 NsiI F	CTAGATGCATATGTGGGTGCGTGCA
GL50803_7188 ApaI R	CTAGGGGCCCTTAAAGAGCTGATAT
GL50803_22587 XhoI F	CTAGCTCGAGATGCCATTAAATCGC
GL50803_22587 PstI R	CTAGCTGCAGTCACTTGTTGGCTCG

**Table S2. List of proteins expressed in *G. intestinalis*.**

1.	GL50803_3491
2.	GL50803_4852
3.	GL50803_7035
4.	GL50803_7188
5.	GL50803_8148
6.	GL50803_10971
7.	GL50803_12229
8.	GL50803_12598
9.	GL50803_14818
10.	GL50803_16386
11.	GL50803_16424
12.	GL50803_16891
13.	GL50803_17276
14.	GL50803_22291
15.	GL50803_22587
16.	GL50803_27910
17.	GL50803_92741



**Table S3. Distribution of the identified proteins in metamonads.**

<i>G. intestinalis</i>	<i>S. salmonicida</i>	<i>E. cyprinoides</i>	<i>C. embranifera</i>	<i>T. vaginalis</i>
<i>GiTim44</i>	-	yes	yes	TVAG_008790
<i>GiMOMP35</i>	-	-	-	-
GL50803_12229	-	-	-	-
GL50803_7035	-	-	-	-
GL50803_8358	-	-	-	-
GL50803_16386	-	-	-	-
GL50803_10971	-	-	-	-
GL50803_4852	-	-	-	-
GL50803_7188	-	-	-	-
GL50803_13922	-	-	-	-
GL50803_22587	-	-	-	-
GL50803_16424	SS50377_12584	-	-	-
GL50803_8148	-	-	-	-
GL50803_17276	-	-	-	-
GL50803_3491	SS50377_15743	-	-	-
GL50803_27910	SS50377_11382	-	-	TVAG_337220 TVAG_462300