Supplementary Information

The small GTPase Arf1 modulates mitochondrial morphology and function

Karin B. Ackema, Jürgen Hench, Stefan Böckler, Shyi Chyi Wang, Ursula Sauder, Heidi Mergentaler, Benedikt Westermann, Frédéric Bard, Stephan Frank, and Anne Spang

SUMMARY OF SUPPLEMANTARY MATERIAL

SUPPLEMENTARY MATERIALS AND METHODS

Yeast plasmids

Pho88 was chromosomally tagged with mCherry using pMaM12-2 (Maeder et al, 2007). The plasmids pVT100U-mtGFP, pYX142-mtGFP, and pVT100U-mt-Rosella have been reported before (Westermann & Neupert, 2000) (Bockler & Westermann, 2014). pYX142-mtRFPm (Kondo-Okamoto et al, 2008), pRS426-FZO1 and pRS426-DNM1 were provided by J. Shaw. pRS416GPD-mtRFP was generated by inserting a pYX142-

mtRFP derived EcoRI/XhoI mtRFP fragment into pRS416GPD. The plasmid expressing spliced HAC1 (pDN390) was supplied by D. T. Ng (Ng et al, 2000).

Mitochondria enrichment and Arf1 differential affinity chromatography

Mitochondria were purified from yeast cultures grown in YP glycerol according to (Meisinger et al, 2006). The Arf1 differential affinity chromatography was performed as described previously (Trautwein et al, 2004) with minor modifications. About 14 mg recombinant ΔN17Arf1Q71L or ΔN17Arf1T31N were immobilized onto NHS agarose (GE healthcare) according to the manufacturer's protocol. About 1,500 OD_{600} of yeast culture per column were converted into spheroplasts (Spang et al, 1998). The spheroplasts were resuspended to 100 OD_{600}/ml in 0.6 M mannitol, 30 mM Tris/HCl pH7.5, 5 mM $Mg(Ac)_2$, 100 mM KCl, 5 mM ß-mercaptoethanol, 1 mM EDTA, 1mM PMSF and disrupted with 15 strokes using an Elvehjem Potter. The lysate was spun 5 min at 1,500 x g to remove unlysed cells, 5 min at 4,000 x g to remove larger membranes and debris, and finally 15 min at 20,000 x g at 4°C. The pellet of the 20,000 x g spin was solubilized in 6 ml 20 mM HEPES/NaOH pH7.4, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.5% TX-100. The extract was spun for 10 min at 20,000 x g and 4°C. The supernatant was incubated with either mock-treated, ΔN17Arf1Q71L-GTP or ΔN17Arf1T31N beads. The guanine nucleotides exchange reactions and washes were performed as described (Trautwein et al, 2004).

Transmission electron microscopy (TEM)

Electron microscopy of worm *C. elegans* oocytes has been performed as described (Ackema et al, 2013; Trzebiatowska et al, 2008). Pictures were taken on a Philips Morgagni 80 KV microscope (Eindhoven, The Netherlands).

Muscle actin staining

RNAi treated TOM70::GFP adult worms were placed in M9 on ice, washed once, fixed in 1 ml of ice-cold methanol for 10 min and briefly sonicated (Hadwiger et al). The worms were allowed to sink by gravity and the methanol was replaced by ice-cold acetone for 5 min. The worms were washed several times with water, stained for 1 h in

PBS containing 8 U/ml rhodamine-phalloidin (Molecular Probes), followed by 3 washes with water, and mounted on a glass slide in CitiFluor (Citifluor Ltd., UK).

Mitotracker staining in *C. elegans* muscles for life cell imaging

Mitochondria were labeled with MitoTracker Red (Invitrogen) as described (Whitten & Miller, 2007) with slight modifications. Worms were placed in deep well slides in 100 µl of M9 buffer to which 1 µl of 1 mM MitoTracker dissolved in DMSO was added. The worms were incubated for 1 hr at RT and recovered on plates containing bacteria for at least 1 hour at RT before imaging.

Paraquat assay

Twenty L1 glp-4 worms were placed on a 3 cm IPTG-MGM plate supplemented with either 0, 0.3 or 0.6 mM paraquat (Sigma) and seeded with bacteria containing a RNAi plasmid against either gbf-1or eat-3 or with no insert (L4440). These plates were incubated at 25^{o}C for five days after which the worms were scored. Four independent experiments, each consisting of triplicates, were scored.

HeLa cells transfection

2.0 x 10^5 HeLa cells were seeded on coverslips coated with poly-L-Lysine in a 6-well dish. After one day's growth, 2 µg (3 µl of 50 µM stock) of each siRNA (siGENOME SMARTpool, Thermo) were taken and mixed with 4 µl of TurboFect (Thermo) transfection reagent in 100 µl DMEM and incubated for 20 min at RT with shaking before applying onto the cells. After 3 days, cells were stained with 0.5 µM of MitoTracker for 30 min at 37°C followed by fixation with 4% paraformaldehyde, 4% sucrose in PBS as well as permeabilization with 0.05% NP-40/PBS. A conventional immunostaining protocol was used.

LatA and benomyl treatment

NMG plates were supplemented with 3.5 nM benomyl or the equivalent volume of DMSO (solvent) in the media. For LatA treatment, 1µl of 24 mM LatA or ethanol

(solvent control) was mixed in to the bacterial lawn. L3 worms were incubated for 2 days on these plates at 20°C.

qPCR

Worms were fed from hatching or L3 (copb-1 and sar-1) to adulthood and lysed in Trizol (Invitrogen). RNA was extracted according to standard procedures as described by the manufacturer. The RNA was cleaned-up further by using Qiagen RNeasy colums. 2 µg of total RNA was digested with DNAse Q1 (Promega) according to the specification sheet. cDNA was made by using Transcriptor reverse transcription (Roche), oligodT (Promega) and RNAsin plus (Promega) following the manufacturer's instructions. Primers used for qPCR: arf-1.2 Q1 fw 5'-CCTCTCCCATCCCTTAGGCT-3', arf-1.2 Q1 re 5'-GACGACAAATGAATTGGGGCT-3', copb1 Q1 fw 5'-AAGGCGACTGTGGATTCCTG-3', copb1 Q re 5'-TTCTCGACAGAGACGTTCGC-3', miro-1 Q1 fw 5'-AGCATTTTTCCCCTTGGTGC-3', miro-1 Q1 re 5'-ATAATTACAGTCGACCCGGCG-3', miro-1/2 Q1 fw 5'-GGGAAGAAGTTGACCAGCGT-3', miro-1/2 Q1 re 5'-TGCCCGTCGAGAACTTGATT-3', sar-1 Q1 fw 5'-GTCCCACTTCATTTCCCCCT-3', sar-1 Q1 re 5'-CGAACTGCAACATTCCGAAGC-3', vdac-1 Q1 fw 5'-CCGATCACTTCCATTCTCCCA-3', vdac-1 Q1 re 5'-TCGACATTGAAAAATCAACGGT-3', Primers for cdc-42 were described before (Hoogewijs et al, 2008). PCR was performed using the Fast start universal sybr green master with ROX (Roche) according to standard recommendations in a StepOnePlus™ Real-Time PCR System. Expression levels were normalized to cdc-42 (Hoogewijs et al, 2008). Fold differences were calculated using the delta-delta Ct method, corrected for PCR efficiency (Pfaffl, 2001). The average knockdown of the target genes was determined based on three independent experiments (Fig. S2). The knockdown efficiency of *gbf-1(RNAi)* and *agef-1(RNAi)* was reported before (Ackema et al, 2013).

Body bend assay

Adult worms were placed in 100 µl M9 buffer into flat bottom 96-well plates, allowed to adapt for 2 minutes before counting the body bends for 1 min. The experiment was repeated with 3 individual feeding experiments with 10 worms each.

SUPPLEMANTARY FIGURE LEGENDS

Figure S1. GBF-1 is essential for mitochondrial function. (**A**) g*bf-1(RNAi)* worms perform poorly in a body bend assay. The experiment was performed three times with 10 worms each. Standard deviation is given. For significance testing, one-way ANOVA was used, followed by a Tukey test ($p<1*10^{-13}$). (**B**) The decrease in mobility of *gbf-1(RNAi)* worms is not due to a disrupted actomyosin system in muscle cells. No differences between mock-treated and *gbf-1(RNAi)* worms were observed when muscle cells were stained for F-actin with rhodamine-phalloidin. (**C**) DIC images of live epifluorescence microscopy merged together using the Photoshop photo merge automation. After a 2-day treatment with LatA or benomyl, worms are severely growth retarded compared to wild-type. (**D**) Live epifluorescence microscopy of TOM70::GFP in *C. elegans* muscle cells treated with either LatA or benomyl and the appropriate solvent control.

Figure S2. Knock-down efficiency of different RNAi constructs. qPCR was performed on adult worms that have been feeding on bacteria expressing the indicated RNAi constructs. The experiments were performed three times. Standard deviation is indicated.

Figure S3. Arf1 does not influence mitochondrial inheritance, but affect mitochondrial function in an allele-specific manner. (**A**) Mitochondrial inheritance is not affected in *arf1-11Δarf2* at 37°C. For each genotype three experiments with >45 cells per experiment were counted. Standard deviation is given. (**B**) Mitochondrial function is affected in *arf1* mutants. *arf1-11*Δ*arf2* showed reduced growth on glycerol compared to glucose. *gea1-19*Δ*gea2* has growth defects on both substrates. (**C**) Mitochondrial morphology is unaltered in *arf1-18*Δ*arf2* cells. Strains transformed with mt-GFP were

grown to early to mid log phase at 23°C. Half of the culture was shifted to 37°C for 1 hr before imaging. (**D**) *arf1-18*Δ*arf2* have functional mitochondria.

Figure S4. Arf1 is not involved in Dnm1 recruitment. Dnm1-GFP is recruited to the mitochondria of *arf1-11Δarf2* yeast cells at the restrictive temperature. As a control we used *Δfis1* cells in which Dnm1 is not recruited to the mitochondria.

Figure S5. Arf1 is localized to mitochondria. (**A**) Different centrifugation purification steps to obtain a mitochondrial enriched fraction. P, Pellet; S, Supernatant. Pgk1 was used as a control for cytoplasm enrichment; Sec61 was used as marker for ER enrichment and Por1 for mitochondrial enrichment. (**B**) The mitochondrial pellet from panel A was used for further fractionation over a sucrose density gradient from which different fractions were collected and analyzed. Arf1 is more strongly enriched in a purified mitochondrial fraction (fraction 8) than in ER fractions (fractions 3 and 4). Representative blots of one of three independent experiments are shown.

Figure S6. (**A**) Cdc48 does not rescue the respiratory growth defects of *arf1-11Δarf2. arf1-11Δarf2* and its isogenic wild-type were grown on glucose or glycerol containing substrates. Over expression of *CDC48* in *arf1-11Δarf2* did not rescue growth defects at either glycerol or on glucose at the restrictive temperature. (**B**) *cdc48* temperature-sensitive mutants do not cause a globular mitochondrial phenotype. A small increase in fragmentation was observed. Cdc48-6, cdc48-3 and a mutant in the Cdc48 co-factor Npl4 expressing mt-GFP were shifted for 1 h to 37°C prior to analysis. For each strain >100 cells were counted. *nlp4* N=156, *cdc48-6* N=115, *cdc48-3* N=119 (**C**) Double RNAi of cdc-48.1 and cdc-48.2 does not cause mitochondrial phenotypes in *C. elegans* muscle cells. Ten worms were counted, with a total of 92 muscle cells. (**D**) Vms1 is not the co-factor required for Cdc48-dependent rescue. Deletion of the potential Cdc48 recruiter *VMS1* did not affect mitochondrial morphology, nor did it affect the globular mitochondrial phenotype of *arf1-11Δarf2. Δ vms1* rescued the fragmented phenotype of *CDC48* overexpression, but did not enhance or reduce the phenotypic rescue of *arf1-11Δarf2CDC48.* Three independent experiments were performed with >100 cells per

experiment. Total cell numbers are *ARF1Δarf2Δvms1* n=360, *arf1-11Δarf2Δvms1* n=335, ARF1*Δarf2 Δvms1CDC48* n=343, *arf1-11Δarf2 Δvms1CDC48* n=328.

Figure S7. (**A**) Artificial tethering of ER and mitochondria does not rescue the mitochondrial phenotype of *arf1-11Δarf2.* Expression of chiMERA did not induce the formation of tubular structures in *arf1-11Δarf2* at the restrictive temperature. The experiment was performed at least three times with at least 80 cells each. Mitochondria are shown in red, the chiMERA construct labeling the ER is shown in green. (**B**) Mdm34-GFP localization is not changed in *arf1-11*Δ*arf2* mutant cells. Mdm34 was chromosomally appended with GFP. Cells were shifted to the restrictive temperature for 1 hr and imaged thereafter.

Movie S1. A body bend assay with WT worms.

Movie S2. g*bf-1(RNAi)* perform poorly in a body bend assay.

SUPPLEMENTARY TABLE S1

Table S1. Mass spectrometric analysis of proteins bound to Arf1-Q71L (GTP) and Arf1-T31N (GDP)

	Arf1-Q71L (GTP)		Arf1-T31N (GDP)	
Position (based on Mascot Score)	Name	# unique peptides	Name	# unique peptides
1	Hsp60	41	Arf1	12
2	Cdc48	40	Pma1	26
3	Imd3	7	eEF1A	22
4	eEF1A	24	Hsp60	23
5	Pma1	28	Pep4	11

SUPPLEMENTARY TABLE S2

Table S2. Yeast strains used in this paper

Strain	Genotype	Source
YPH499	MATa *ura3-52 leu2*-D*1 trp1*-D*63 his3*-D*200 lys2-801 ade2-101*	(Sikorski & Hieter, 1989)
YPH500	MATα *ura3-52 leu2*-D*1 trp1*-D*63 his3*-D*200 lys2-801 ade2-101*	(Sikorski & Hieter, 1989)
CJY049-3-4	MATa *ura3-52 leu2-3,112 his3*-D*200 lys2-801 ade2-101*	C.L. Jackson
APY 022	MATα *ura3-52 leu2-3,112 his3*-D*200 gea1-6 gea2*::*HIS3*	C.L. Jackson
APY 026	MATa *ura3-52 leu2-3,112 his3*-D*200 gea1-19 gea2*::*HIS3*	C.L. Jackson
NYY0-1	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::ARF1::ADE2*	(Yahara et al, 2001)
NYY11-1	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::arf1-11::ADE2*	(Yahara et al, 2001)
YAS1682	MATa, *ura3-52 leu2*-D*1 trp1*-D*63 his3*-D*200 lys2-801 ade2-101 sec27-1*	(Erdeniz et al, 1997)
YAS1683	MATa, *ura3-52 leu2*-D*1 trp1*-D*63 his3*-D*200 lys2-801 ade2-101 sec21-1*	(Erdeniz et al, 1997)
YAS1834	MATa, *ura3-52 leu2*-D*1 trp1*-D*63 his3*-D*200 lys2-801 ade2-101 sec23-1*	(Erdeniz et al, 1997)
YAS4036	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::ARF1::ADE2 PHO88::PHO88-3x mCherry-Kan*	This study
YAS4037	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::arf1-11::ADE2 PHO88::PHO88-3x mCherry-Kan*	This study
YAS4040	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::ARF1::ADE2 fis1::Kan*	This study
YAS4079	MATa *ura3 lys2 trp1 leu2 arf1::HIS3 arf2::HIS3 ade2::ARF1::ADE2 Kan-GPD::CDC48 FZO1-FLAG-TRP*	This study
YAS4080	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3 ade2::arf1-11::ADE2 Kan-GPD::CDC48 FZO1-FLAG-TRP*	This study
YAS4084	MATa *ura3 lys2 trp1 leu2 arf1::HIS3 arf2::HIS3 ade2::ARF1::ADE2 Kan-GPD::CDC48 FZO1-GFP-TRP*	This study
YAS4083	MATa *ura3 lys2 trp1 leu2 arf1 ::HIS3 arf2::HIS3*	This study

	ade2::arf1-11::ADE2 Kan-GPD::CDC48 FZO1-GFP-TRP mt-RFP (LEU)	
RJD3411	MATa *cdc48-3 leu2-3 his3-11,-15 trp1-1 ura3-1 ade2-1*	R. Deshaies
RJD3092	MATα *his3-Δ200 leu2- 3112 lys2- 801 trp1- 1, ura3- 52 cdc48- 6*	R.Deshaies/M. Hochstrasser
RJD2589	MATa *ura3-52 leu2Δ1 trp1Δ63, npl4- 1*	R. Deshaies/P. Silver

References Supplementary Material

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