

Charcot-Marie-Tooth disease associated mutants of GDAP1 dissociate its roles in peroxisomal and mitochondrial fission

Running title:
GDAP1 in peroxisomal fission

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Summary

Mitochondria and peroxisomes can be fragmented by the process of fission. The fission machineries of both organelles share a set of proteins. GDAP1 is a tail-anchored protein of mitochondria and induces mitochondrial fragmentation. Mutations in *GDAP1* lead to Charcot-Marie-Tooth disease (CMT), an inherited peripheral neuropathy, and affect mitochondrial dynamics. Here, we show that GDAP1 is also targeted to peroxisomes mediated by the import receptor Pex19. Knockdown of GDAP1 leads to peroxisomal elongation that can be rescued by re-expressing GDAP1 and by missense mutated forms found in CMT patients. GDAP1-induced peroxisomal fission is dependent on the integrity of its hydrophobic domain 1, and on Drp1 and Mff, as is mitochondrial fission. Thus, GDAP1 regulates mitochondrial and peroxisomal fission by a similar mechanism. However, our results reveal also a more critical role of the N-terminal GDAP1 domains, carrying most CMT-causing mutations, in the regulation of mitochondrial compared to peroxisomal fission.

Keywords: glutathione *S*-transferase/ mitochondrial dynamics/ peripheral neuropathy/ tail-anchored protein/

Introduction

Peroxisomes are dynamic, single membrane-bound organelles. They are responsible for several metabolic processes such as fatty acid β -oxidation, biosynthesis of ether phospholipids, and metabolism of reactive oxygen species (ROS) [1]. Numbers, composition, and morphology of peroxisomes are influenced by extra- and intracellular stimuli [2]. Peroxisomes are generated by two different mechanisms: They can form *de novo* from the endoplasmic reticulum or by growth and division from pre-existing peroxisomes. The contribution of the two pathways to peroxisome biogenesis is a matter of intensive debate [3-5].

Peroxisomal growth and division occurs in morphologically well defined steps: The spherical peroxisome first elongates in response to exogenous or endogenous stimuli mediated by Pex11 proteins [4]. Next, the membrane of the elongated peroxisome forms constrictions by a not yet clearly defined mechanism [6]. Final fragmentation requires several fission proteins, including the tail-anchored (TA) proteins hFis1 (Fission 1), Mff (Mitochondrial fission factor) and the cytosolic Drp1/DLP1 (Dynamin-related protein 1) [7-12]. These proteins are also essential fission factors at the mitochondrial outer membrane (MOM). Sharing of fission components has probably developed by similar cellular demands as peroxisomes and mitochondria are metabolically linked [13]. This cooperative interaction likely influences the functionality of both organelles in health and disease [14,15].

GDAP1 (ganglioside-induced differentiation associated protein 1) is also a TA-protein of the MOM acting as mitochondrial fission factor [16-18]. GDAP1 is the founder of a family of glutathione *S*-transferases (GST) [19] and its expression level influences glutathione levels in cultured cells [20]. Over 40 different mutations in *GDAP1* lead to Charcot-Marie-Tooth disease (CMT), the most commonly inherited peripheral neuropathy [21,22]. Recessively inherited disease mutants (rmGDAP1) show reduced mitochondrial fragmentation activity, whereas dominantly inherited disease mutants (dmGDAP1) interfere with mitochondrial fusion. This impaired fusion results in a disturbed mitochondrial membrane potential and increased ROS levels [17].

The aim of this study was to investigate the possible role of the TA-protein GDAP1 as fission factor at peroxisomes and its relevance in disease. We show that GDAP1 is targeted to a subpopulation of peroxisomes in a Pex19-dependent manner. Loss of GDAP1 leads to peroxisomal elongation, whereas overexpression increases peroxisomal fragmentation. Disease-mutated forms of GDAP1 can still induce peroxisomal fragmentation with comparable efficiency unless the mutation interferes with peroxisomal targeting via its TA.

GDAP1-induced peroxisomal fragmentation is dependent on Drp1, Mff, and requires the hydrophobic domain 1 (HD1) lying N-terminally to the transmembrane domain (TMD) of GDAP1 [18]. We conclude that GDAP1 induces fission at the MOM and at the peroxisomes by overlapping mechanisms. However, the finding that N-terminal rmGDAP1 missense mutants retain peroxisomal fission activity, while losing this activity on mitochondria, revealed also mechanistic differences.

Results and Discussion

GDAP1 is targeted to peroxisomes

TA-proteins with positively charged amino acids surrounding the C-terminal TMD are inserted into the MOM and can be inserted into the peroxisomal membrane of mammalian cells [23]. As GDAP1 is a TA-protein with such features [18], we tested its co-localization with peroxisomal markers. GDAP1 was transiently expressed in COS7 cells stably expressing GFP-SKL, a GFP with a C-terminal peroxisomal targeting sequence [24]. As expected, GDAP1 staining predominantly co-localized with the mitochondrial marker cytochrome *c* [16]. Additionally, some peroxisomes co-localised with the GDAP1 signal (Fig.1A). We also analysed endogenous GDAP1 in mouse primary hippocampal cell cultures infected with lentivirus encoding GFP-SKL. The signal of endogenous GDAP1 protein predominantly co-localized with cytochrome *c* and partially with GFP-SKL-positive peroxisomes (Fig.1B). Taken together, GDAP1 shows dual targeting to mitochondria and peroxisomes.

TA-proteins are targeted to peroxisomes via the Pex19 shuttle receptor [25-28]. In an interaction assay, *in vitro* translated GFP-GDAP1 interacted with recombinant Pex19 and formed a trimeric complex with Pex19 and Pex3 (Fig.S1A). To confirm the functional relevance of this interaction we infected COS7 cells stably expressing GFP-SKL with lentiviruses encoding two different shRNAs targeting Pex19 or a non-silencing control shRNA. When reduced expression of Pex19 was significant (Fig.S2A), cells were transiently co-transfected with GDAP1 and mitochondrially-targeted DsRed2 (mtDsRed) expression constructs. After 16 h, cells were fixed, immunostained for GDAP1, and the signal intensity of GDAP1 was determined at peroxisomes (GFP-SKL-positive structures) and mitochondria (mtDsRed-positive structures) on single plane confocal images (Fig.S1B). The GDAP1-signal intensity at peroxisomes was significantly lower in Pex19 knockdown cells compared to controls. Under the same conditions, targeting of GDAP1 to mitochondria was not affected (Fig.1C). We conclude that GDAP1 is targeted to peroxisomes via the peroxisomal import receptor Pex19.

Loss of GDAP1 reduces peroxisomal fragmentation

To assess the functional relevance of GDAP1 at peroxisomes, we infected mouse neuroblastoma N1E-115 cells with lentiviruses encoding two different shRNAs against GDAP1. Knockdown of Drp1 by two different shRNAs served as positive controls, while a non-targeting shRNA and uninfected cells were used as negative controls. Five days after infection, cells were fixed and stained for Pex14 as peroxisomal marker. Sister plates were

analysed by immunoblotting to confirm the knockdowns (Fig.S2B,C). To quantify the morphologies, we distinguished three different categories: spherical peroxisomes, elongated peroxisomes, and tubular peroxisomes (Fig.2A). Uninfected and control-infected cells generally showed a spherical peroxisomal morphology. Reduced levels of GDAP1 led to elongated peroxisomes. This effect was even more pronounced after knockdown of Drp1, where most peroxisomes were tubular as described (Fig.2A) [6,8]. This experiment demonstrated that loss of GDAP1 alters peroxisomal morphology. Peroxisomal proliferation can be mediated by elongation of existing peroxisomes. Elongated peroxisomes get constricted and subsequently fragmented by the fission machinery [14]. Therefore, both reduced fission activity and enhanced elongation may shift the overall peroxisomal morphology towards a more tubular, less spherical phenotype. GDAP1 expression levels influence glutathione (GSH) levels in cultured cells [20], thereby potentially influencing peroxisomal biogenesis by increasing elongation due to cellular stress. Thus, we measured GSH and ROS levels in acute GDAP1 knockdown cells by the enzymatic recycling method and 2,7-dichlorofluorescein (DCF) fluorescence intensity measurements, respectively [17,29]. Cellular GSH levels after GDAP1 knockdown by two individual shRNAs showed no significant difference to control cells (Fig.2B). As positive control, uninfected cells were treated with ethacrynic acid (EA), which led to significant GSH depletion. DCF fluorescence intensities were also identical between GDAP1 knockdown cells and controls, while EA-treatment increased DCF fluorescence intensity (Fig.2C). In summary, short-term knockdown of GDAP1 did not affect intracellular GSH levels or cause ROS stress in cultured cells and cannot account for the peroxisomal elongation due to cellular stress. Thus, our results indicate that the observed morphological changes of peroxisomes in GDAP1 knockdown cells are caused by reduction in fission activity.

To further test the impact of GDAP1 on fission, we made use of transient Pex11 β -myc overexpression, which elongates peroxisomes independent of other stimuli [6,30,31]. In N1E-115 cells the strongest elongation effect can be observed seven hours after transfection. Subsequently, the endogenous fission machinery of the cell is activated; in consequence 24 h after Pex11 β -myc transfection cells predominantly exhibit a spherical peroxisomal morphology, which represents the new equilibrium of forced elongation and endogenous fission events (Fig.S3). Also in this paradigm, knockdown of GDAP1 resulted in less cells with spherical peroxisomes 24 h after Pex11 β -myc transfection, as did knockdown of the well-established fission factor Drp1 (Fig.S4A,B), supporting that GDAP1 acts as peroxisomal fission factor.

Dominantly and recessively inherited GDAP1 missense disease mutants can promote peroxisomal fragmentation

We have previously rescued mitochondrial fission after GDAP1 knockdown by re-expressing GDAP1 enabling us to examine also disease-linked mutants [17]. This experimental paradigm was adapted for the analysis of peroxisomal fragmentation. Four days after lentiviral infection with control or GDAP1-targeted shRNAs, N1E-115 cells were transiently co-transfected with Pex11 β -myc and expression vectors encoding mitochondrially targeted eGFP (mtGFP) as control, GDAP1 wildtype, dmGDAP1, or rmGDAP1 missense mutants (Fig.3A). Mutants covering different domains of the protein were selected (Fig.S5A). Twenty-four hours after transfection, when tubulation by Pex11 β -myc-overexpression is counterbalanced by active fission (Fig.S3), cells were fixed and morphologically analysed. Uninfected and control infected mtGFP and Pex11 β -myc expressing cells showed predominantly spherical peroxisomes. GDAP1 knockdown resulted in more cells with elongated peroxisomes as fission is decreased (Fig.3B,C). Re-introduction of wildtype GDAP1 into these knockdown cells restored the peroxisomal morphology to control levels, validating the experimental setup and GDAP1's role as fission factor. Overexpression of all tested missense disease mutants in GDAP1 knockdown cells also reconstituted the peroxisomal morphology (Fig.3B,C). Wildtype GDAP1, tested dmGDAP1s or rmGDAP1s showed no significant differences in their ability to induce peroxisomal fragmentation. This was unexpected, as disease-causing mutant forms of GDAP1 influence mitochondrial dynamics (Tab.SI; [16,17]).

To confirm this finding, we expressed Pex11 β -myc in N1E-115 cells overexpressing GDAP1 wildtype, dmGDAP1, or rmGDAP1 missense mutants and analysed peroxisomal morphology seven hours after start of transfection (Fig.S5B). In this setting, an active fission factor will counterbalance the Pex11 β peroxisome elongation effect, which is maximal at this time point in control cells (Fig.S3). All tested disease-associated missense mutants were able to induce fragmentation of Pex11 β -myc stimulated peroxisomes with comparable efficiency as GDAP1 wildtype, resulting in an overall spherical morphology (Fig.S5C). In summary, in both experimental approaches (Fig.3B,C and S5C) we found that the disease-associated missense mutations do not impair the peroxisomal fission capacity of GDAP1. As mitochondrial fission activity of rmGDAP1 missense mutants is reduced [16,17], our results further indicate that mutations in N-terminal domains differently affect GDAP1's activity at mitochondria versus peroxisomes.

The hydrophobic domain 1 critically regulates GDAP1-induced peroxisomal fission

Two domains are required for mitochondrial fission: the hydrophobic domain 1 (HD1) and the C-terminal TA-domain, which mediates mitochondrial targeting (Fig.S5A, Tab.SI) [18]. To examine whether mitochondrial and peroxisomal fission induction by GDAP1 depend on the integrity of the same domains, we used the experimental setting as described before in Fig.3A. We tested GDAP1 constructs coding for a protein with a scrambled HD1 (HD1scr), a deleted HD1 (Δ HD1) or C-terminal truncated GDAP1. The mutant hT288X corresponds to the largest GDAP1 truncation described in CMT patients lacking the HD1 and TA-domain, and hT318X is an artificial truncation mutant lacking only the TMD and the C-terminal residues [14,15]. None of these mutants was able to induce peroxisomal fragmentation (Fig.3D,E). These results were confirmed using the experimental settings as described in Fig.S5B (Fig.S5D). Thus, the TA-domain and the HD1 are essential for fission at peroxisomes as well as at mitochondria. Mutations in *GDAP1* interfering with targeting are associated with more severe clinical phenotypes than point mutations leading to amino acid exchanges in the N-terminal part of GDAP1 [32]. Our results correlate with the different severities and imply that disease-associated missense mutations in the N-terminal part of GDAP1 affect exclusively mitochondrial dynamics, while loss or C-terminal truncation of GDAP1 additionally affect peroxisomal dynamics (Tab.SI).

GDAP1-induced peroxisomal fission is dependent on Drp1 and Mff

The previous data confirm that GDAP1-dependent fragmentation requires the intact HD1 and proper protein targeting to the peroxisomal membrane as for mitochondrial fission. Next, we investigated whether GDAP1-induced peroxisomal fission is dependent on Drp1, the executive GTPase in mitochondrial and peroxisomal fission [14,15] and on Mff, the adaptor protein for Drp1 at peroxisomes and mitochondria in mammalian cells [10,12]. We used Drp1 or Mff knockdown (Fig.S2C,D) in combination with Pex11 β -myc and GDAP1 overexpression, as we did before in GDAP1 knockdown cells (Fig.3A). N1E-115 cells were infected with lentiviruses encoding non-targeting control shRNA, Drp1, or Mff shRNA constructs, followed by co-transfection with expression vectors coding for Pex11 β -myc and mtGFP as control, or for Pex11 β -myc and GDAP1. The next day, cells were fixed and morphologically analysed. As expected, Pex11 β -myc expression together with control mtGFP led to highly tubular peroxisomes in Drp1 knockdown cells (Fig.4A,B). Overexpression of combined Pex11 β -myc and GDAP1 in Drp1 knockdown cells resulted in an identical tubular peroxisomal morphology. Similarly, knockdown of Mff led to highly tubular peroxisomes

(Fig.4C,D). In the absence of Mff, GDAP1 expression could not alter the peroxisomal morphology, demonstrating that GDAP1 cannot substitute for Mff in peroxisomal fission. We also analysed the mitochondrial morphology in N1E-115 cells with Mff knockdown and found that GDAP1-induced mitochondrial fission is as well dependent on Mff (Fig.S6). Thus our data show that GDAP1-induced peroxisomal fission, like mitochondrial fission, is dependent on Mff and Drp1 [17].

Concluding remarks

Existing peroxisomes can elongate and are subsequently fragmented by the peroxisomal fission machinery to generate new peroxisomes. Here we show that the mitochondrial fission factor GDAP1 is also a peroxisomal fission factor. Loss of GDAP1 results in elongated peroxisomal morphologies, as the fission capacity is decreased, whereas overexpression promotes peroxisomal fragmentation. GDAP1-induced fission relies on the presence of Mff and Drp1, demonstrating that GDAP1 influences fission upstream of the conserved basic fission machinery of mitochondria and peroxisomes. GDAP1 is not ubiquitously expressed, however, its accessory function is essential for myelinated peripheral nerves as mutations in *GDAP1* lead to CMT. To induce fission, the integrity of HD1 is essential in GDAP1. Consistently, truncations lacking this domain or the targeting tail-anchored domain have lost the ability to promote peroxisomal fission. All other disease mutations in the N-terminal cytosolic GDAP1 domain are still able to promote peroxisomal fragmentation. These findings stand in contrast to our previous observations at the MOM, where the fission-induction function of these recessively inherited missense mutants is reduced [16,17], suggesting different regulatory mechanisms for GDAP1-induced fission at both organelles. Furthermore, our results reveal a difference in the cell biology of CMT-associated N-terminal missense mutations and the more severe C-terminal truncation mutations of GDAP1.

Methods

Constructs

All constructs for transient transfections and lentiviral vectors and production of viruses have been described [16-18, 27, 31]. Lentiviruses expressing shRNA targeting Mff or Pex19 were purchased from Sigma (clone ID: NM_029409.2-484s1c1, NM_029409.2-693s1c1; NM_002857.2-88s1c1, NM_002857.2-90s1c1). MtDsRed and GFP-SKL were inserted into a modified pSicoR backbone (Addgene) containing the CMV promoter and multiple cloning site from the pcDNA3.1 vector (Invitrogen).

Cell culture

Hippocampi of P1 C57BL/6J mice (Janvier, France) were dissected, trypsinized, and triturated. 50.000-100.000 cells /ml were seeded on Matrigel (BD Bioscience) coated cover slips in Neurobasal medium (Gibco) supplemented with B27 (Gibco), 50 ng/ml NGF (Harlan), 2 mM L-Glutamine (Gibco), 4 mg/ml D-Glucose (Sigma). Mouse primary hippocampal neurons were infected with lentiviruses one day after preparation and kept in culture for 4-6 days. COS7 cells stably expressing GFP-SKL, HEK-293T cells, and N1E-115 cells were maintained, transfected and infected as described previously [17].

Immunocytochemistry

Immunocytochemistry was described [16]. For co-staining of GDAP1 and Pex11 β -myc, cells were permeabilized with Digitonin (2.5 μ g/ml, 5 min) and stained using antibodies against myc, followed by a permeabilization with 0.2 % Triton X-100 for 10 min and subsequently stained for GDAP1. Antibodies: anti-GDAP1 [16], anti-human GDAP1 [35], anti-Pex14 (Proteintech Europe), anti-cytochrome *c* (Pharmingen), anti-myc (clone 9E10).

Biochemical Methods

Expression and purification of recombinant proteins and native polyacrylamide gel electrophoresis was performed as described [27]. The measurement of GSH levels and DCF fluorescence in cells has been performed according to [17,29]. Western blotting, detection and quantification was performed as described [16]. Antibodies: anti-GDAP1 [16], anti-Dlp1 (BD Bioscience), anti-Pex19 (Sigma), anti-Mff (A. van der Bliek), anti-GAPDH (HyTest), and anti- β -actin (Sigma).

Acknowledgements

The authors thank Alexander van der Blik for kindly providing reagents, Michèle Telorack for help in performing the GSH measurement, Claudia Grou and Manuel Pinto (IBMC, Porto) for support in *in vitro* translation assay and Ned Mantei for critically reading the manuscript. We thank the Light Microscopy Centre of the ETH Zürich. This work was supported by the Swiss National Science Foundation and the National Center for Competence in Research (NCCR) Neural Plasticity and Repair (to U.S.), BBSRC (BB/K006231/1), and the Portuguese Foundation for Science and Technology (FCT) and FEDER (PTDC/SAU-OSM/103647/2008; PTDC/BIA-BCM/118605/2010 to M. S.; SFRH/BD/73532/2010 to S. G.).

Author Contribution:

N.H. was the primary experimentalist, contributed to the study design and writing of the manuscript. S.G. and A.N. contributed experimental work. M.S., U.S. and A.N. conceived and directed experiments and wrote the manuscript.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1

GDAP1 is targeted to peroxisomes. (A) COS7 cells stably expressing GFP-SKL were transfected with GDAP1 expression constructs and stained for GDAP1 and cytochrome *c*. GDAP1 is mainly localised to mitochondria. Higher magnification (zoom box) shows GFP-SKL-positive structures that are positive for GDAP1 but negative for cytochrome *c* (arrows). A neighbouring, untransfected cell (light yellow broken line). (B) Endogenously GDAP1-expressing primary hippocampal cell cultures were infected with lentivirus encoding GFP-SKL, and stained for GDAP1 and cytochrome *c*. Higher magnification (zoom box) shows that endogenous GDAP1 predominantly co-localizes with the cytochrome *c*, and also with GFP-SKL-positive structures, which are negative for cytochrome *c* (arrows). (C) Measured GDAP1 fluorescence intensity in the peroxisomal population was decreased in Pex19 knockdown cells but unaffected in the mitochondrial population. Values represent means and s.e.m. of three independent experiments. Fluorescence intensity of the peroxisomal/mitochondrial population was analysed for 6 to 9 cells per condition per experiment: * $p < 0.05$, two-tailed unpaired t-test. Bars, 10 μm .

Figure 2

Loss of GDAP1 leads to peroxisomal elongation. (A) N1E-115 cells were infected with lentiviruses encoding shRNA against GDAP1, Drp1 or a non-targeting control (ctrl) shRNA, or were left uninfected. Five days after infection, peroxisomal morphologies were assessed after immunostaining for Pex14 according to the three categories spherical, elongated or tubular and were quantified in blinded countings. (B) Measurements of cellular GSH levels and (C) ROS levels show no significant alterations at day five of the GDAP1 knockdown compared to control cells. Ethacrynic acid (EA) treated cells served as positive control (50 $\mu\text{g/ml}$ for 2 h). Values represent means and s.e.m. of three independent experiments. For morphology analysis, 100 cells were counted per condition per experiment: * $p < 0.05$, ** $p < 0.01$, two-tailed unpaired t-test. Bars, 2.5 μm .

Figure 3

Peroxisomal fission is dependent on the GDAP1 targeting domain and the HD1, but unaffected by CMT-disease associated N-terminal missense mutations. (A) N1E-115 cells were infected with lentiviruses encoding shRNA against GDAP1 or a non-targeting control (ctrl) shRNA or left uninfected (d0). After four days (d4), cells were co-transfected with Pex11 β -myc and GDAP1 wildtype, mutant forms of GDAP1, or mtGFP. 24 h later (d5), cells

were fixed and stained (C,E). Peroxisomal morphology of double-positive cells was quantified in a blinded counting (B,D). (GDAP1 with scrambled HD1 (HD1 scr), GDAP1 with deleted HD1 (Δ HD1), C-terminal truncated human GDAP1 (hT288X, hT318X)). Values represent means and s.e.m. of at least three independent experiments, 100 cells were counted per condition per experiment: * $p < 0.05$, ** $p < 0.01$, two-tailed unpaired t-test. Bars, 10 μ m.

Figure 4

GDAP1-induced peroxisomal fragmentation depends on Drp1 and Mff. The experiment was performed as illustrated in Fig.3A. Knockdown of Drp1 (A,B) or Mff (C,D) in combination with co-expression of Pex11 β -myc and GDAP1 or mtGFP result in tubular peroxisomes. Values represent means and s.e.m. of three independent experiments, 100 cells were counted per condition per experiment: ** $p < 0.01$, two-tailed unpaired t-test. Bars, 10 μ m.

Figure 1

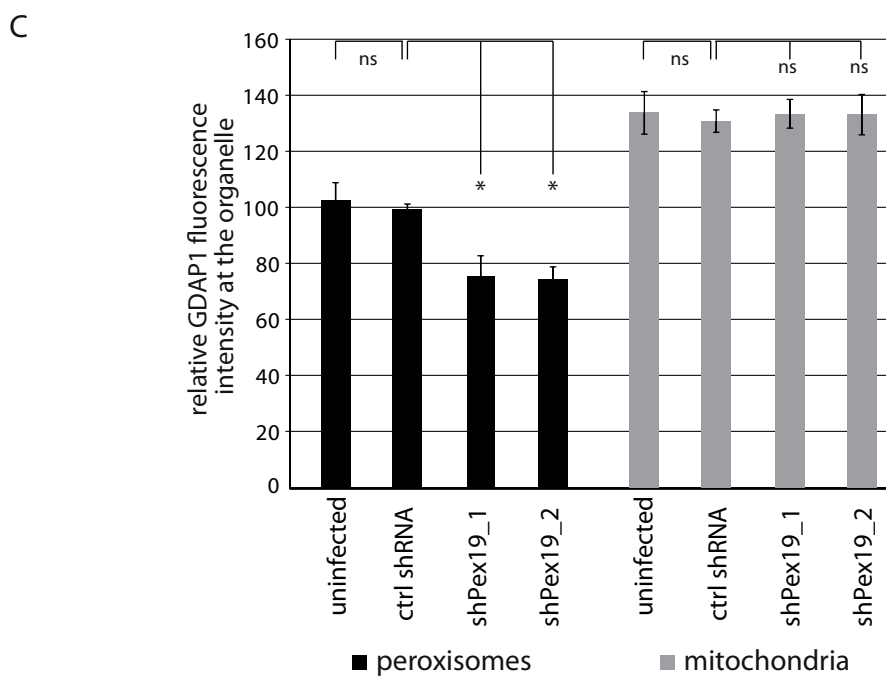
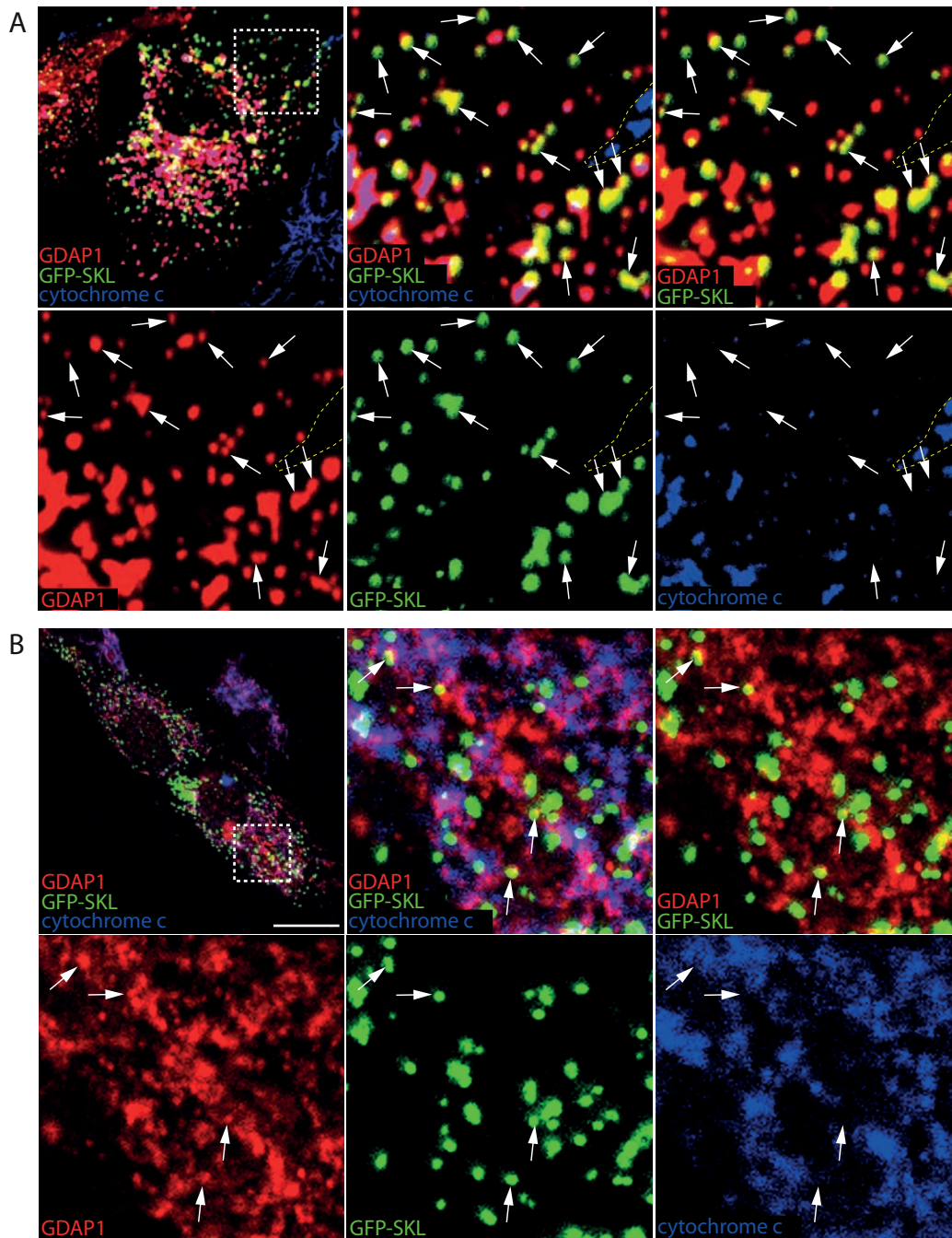


Figure 2

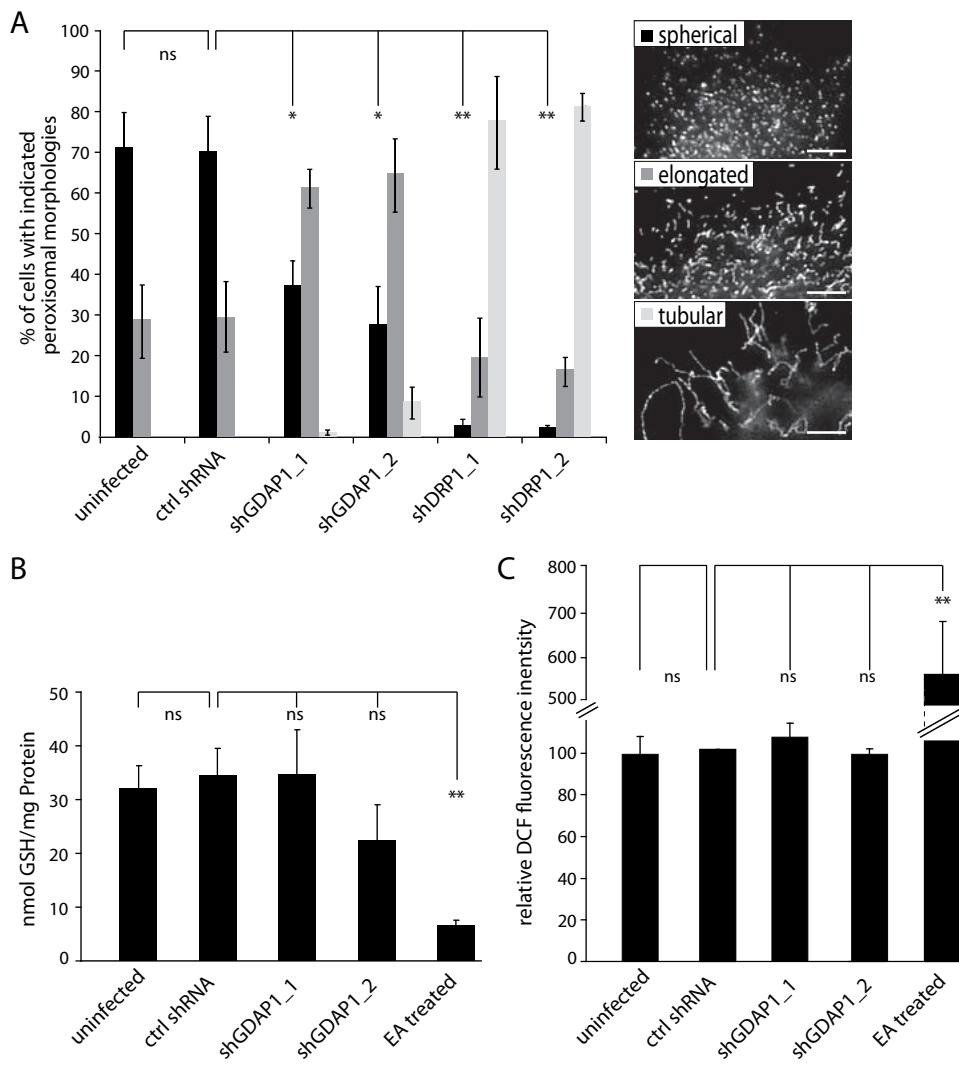


Figure 3

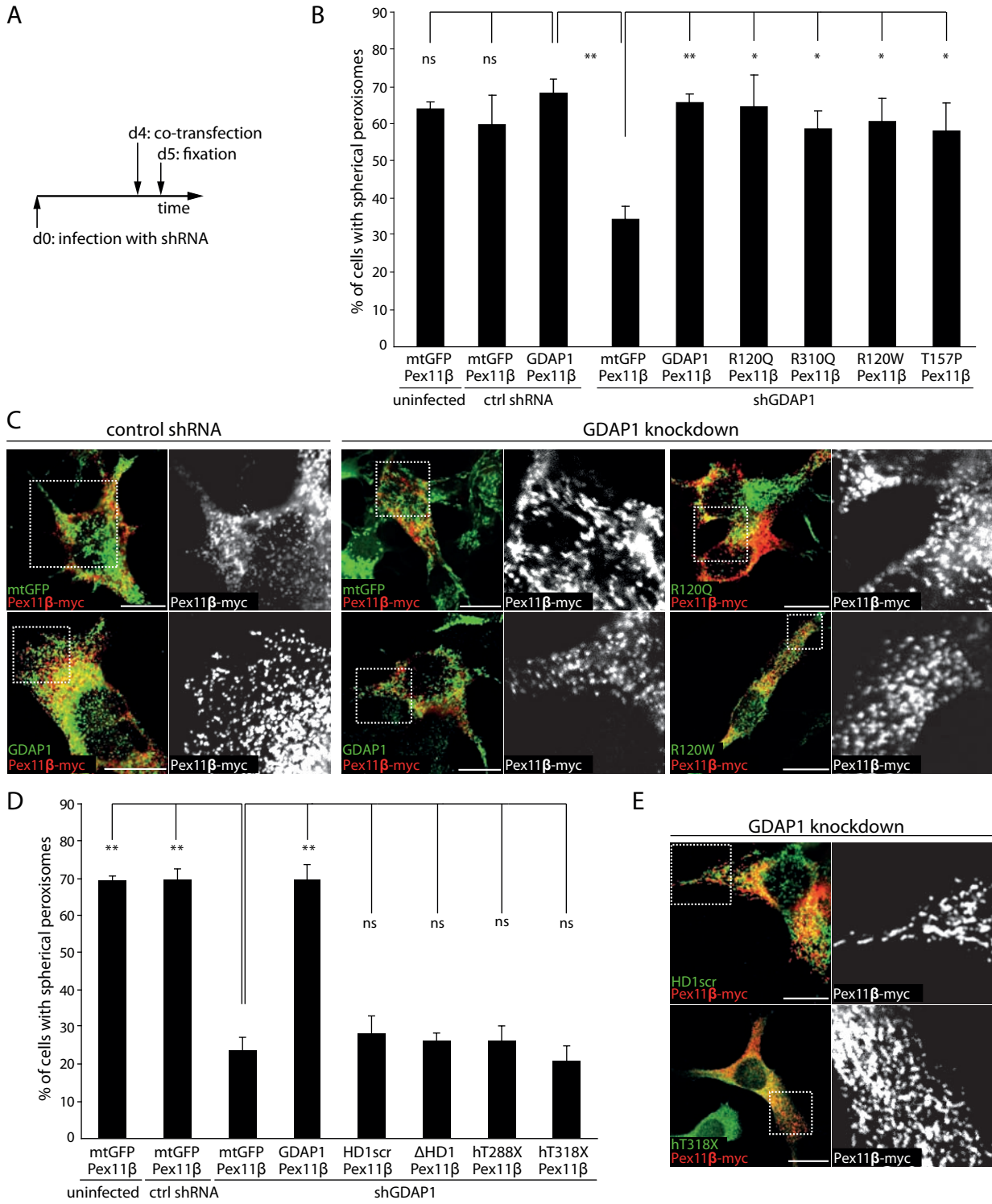


Figure 4

