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1. INTRODUCTION

1.1. MITOCHONDRIA

Mitochondria (derived from the Greek words *mitos* for thread and *khondrion* for small grain) are 0,5 – 1 µm big organelles that are present in most eukaryotic cells [1]. They are derived from eubacterial ancestors [2] and are composed of an outer membrane (OM), an inner membrane (IM) with invaginations called cristae and the matrix [3].

In the cell, mitochondria fulfill a variety of important functions: ATP generation through respiration for energy supply [2], calcium signaling, oxidation of fatty acids, lipid biosynthesis, haem biogenesis, cellular homeostasis, mitochondrial DNA maintenance and stress-induced and developmental apoptosis [3,4].

Mitochondria are not static; they migrate within cells via microtubules and undergo continuous cycles of fission and fusion thereby forming a network-like structure. This process is called mitochondrial dynamics and enables cells to adapt to changing energy demands.

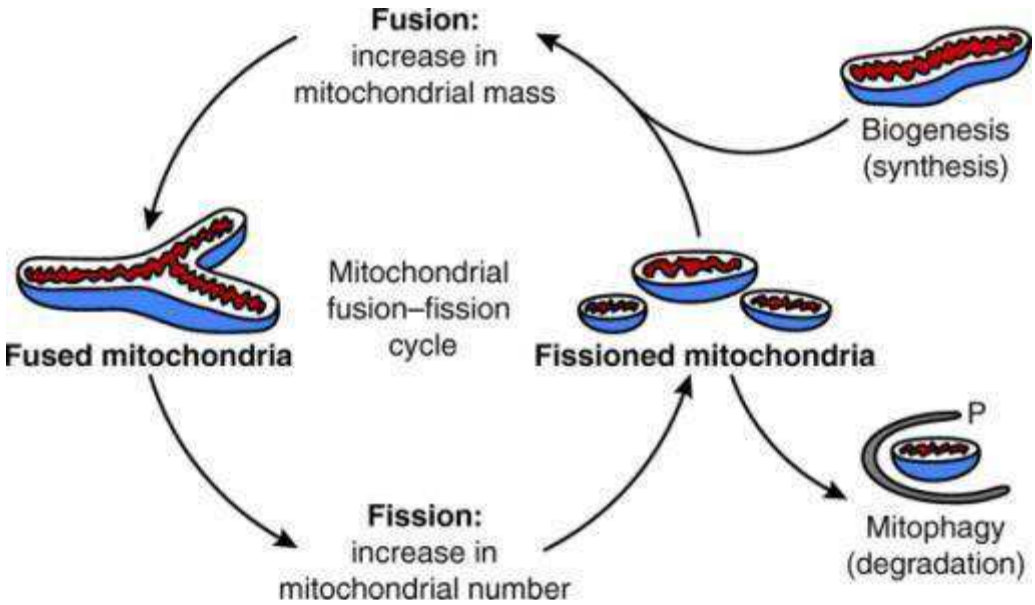


Figure 1. Possible relationship between mitochondrial fusion, fission, biogenesis and degradation; adapted from Seo et al, 2010 [5].

Fission and fusion ensure that mitochondria are well distributed, both inside cells (in distal parts and especially at sites where ATP is required) and during cell division [2, 4, 6]. Moreover, fission causes degradation of defective sections and dysfunctional or misfolded proteins [7]. These very important processes, fission and fusion, are mediated by large, highly-conserved GTPases that belong to the dynamin superfamily.

1.2. DYNAMIN-LIKE PROTEINS AND THEIR ROLE IN MITOCHONDRIAL DYNAMICS

1.2.1. THE FUSION MACHINERY

A. INNER MEMBRANE FUSION

Human OPA1 (optic atrophy 1) and its yeast ortholog Mgm1 mediate fusion of the IM and cristae organization [4]. Both proteins are targeted to mitochondria by a mitochondrial targeting sequence (MTS) and are anchored to the IM with one transmembrane helix so that the GTPase domain points into the intermembrane space. The MTS is cleaved afterwards, producing the so-called L-form which is further processed into the S-form [4, 8]. In fact, various different isoforms are known that are formed by alternative splicing and proteolysis [3]. Regulation of IM fusion also involves a protein family called prohibitins [4].

B. OUTER MEMBRANE FUSION

Human mitofusins (Mfn1 and Mfn2) and yeast ortholog Fzo1 are located at the OM and mediate its fusion. Both mitofusin proteins can form homo- or heterocomplexes which tether the membranes via an interaction of their coiled-coil domains together [3, 8].

Mfn1 shows a higher GTPase activity than Mfn2 and is believed to mediate initial steps of the fusion process. Mfn2, however, shows a higher affinity for GTP and might be involved in regulation of the fusion complex assembly [2].
In 2006, mitofusin binding protein (MIB) was identified as a negative regulator: The 55 kDa protein blocks GTPase activity of Mfns by binding to their GTPase domain via its co-enzyme binding domain [3, 4]. Other regulatory proteins are Bax and Bak, two pro-apoptotic members of the Bcl-2 (B cell lymphoma-2) family. In healthy cells, these proteins stimulate fusion, whereas in cells that undergo apoptosis, activation of Bax and Bak leads to inhibition of fusion [3]. Up to now, it is not clear whether the fusion processes of the inner and the outer membrane are coupled or independently executed [3].

1.2.2. THE FISSION MACHINERY

A. IN YEAST: DNM1, FIS1 AND MDV1

In yeast, mitochondrial fission is catalyzed by Dnm1 (dynamin-like protein 1), which is localized in the cytosol and upon self-assembly on the mitochondrial OM.
Fis1, an 18 kDa protein, that is distributed throughout the OM of mitochondria, is believed to function as a membrane receptor which recruits Dnm1 assemblies and adaptor proteins Mdv1 and Caf4 to the membrane [3]. These interactions might be mediated by the cytosolic part of Fis1, which is considered to serve as a platform for protein interactions. The 80 kDa protein Mdv1 colocalizes and interacts with Dnm1 at mitochondrial fission sites and possibly facilitates division through promoting Dnm1 assembly. The function of Caf4, a non-essential adaptor protein, is unknown. Interestingly, fission does not occur at all sites of the OM that are associated with Mdv1/Dnm1 complexes.
Since all proteins mentioned above are localized to the OM, it is believed that an independent inner membrane division machinery exists. Mdm33, an IM-protein, has been identified as a possible mediator [2, 4].

B. IN HUMANS: DRP1

The main mitochondrial division factor DRP1 exists as a tetramer in the cytosol; only 3% are associated with mitochondria [1, 6, 7, 9]. Upon apoptotic stimuli and Ca^{2+} release, DRP1 is targeted to the OM of mitochondria where it forms mitochondrial foci [3]. Interestingly, not at all foci fission occurs [4].

The mechanism of DRP1 translocation to the mitochondrial OM is still unclear and up to now, orthologs of the yeast Mdv1 or Caf4 have not been identified in higher eukaryotes [2].

So far it is believed that hFis, an integral OM protein, targets DRP1 through interaction with a yet unknown adaptor protein [4]. hFis is localized throughout the OM of mitochondria, predominantly in ganglioside rich microdomains [3]. Recent studies have shown that knockdown of hFis does not interfere with DRP1 localization. Currently, an ortholog of the OM protein Mff (mitochondrial fission factor in Drosophila), which is predominantly located at the OM, is assigned to regulate fission by acting as a DRP1 receptor. This theory further states that DRP1 self-assembles on Mff-bound sites of mitochondria and that hFis acts as an adaptor protein [4, 10].

Studies of DRP1 mutants that cannot bind GTP (K38A) showed that GTP binding is necessary for DRP1 assembly and translocation to mitochondria [2,4].

Membrane constriction requires GTP hydrolysis that induces conformational changes that ultimately lead to mitochondrial division.

In the absence of DRP1, fission of the IM occurs independently. So far, however, no Mdm33 ortholog has been found in higher eukaryotes [2].

One possible component of this inner membrane division machinery may be the IM-protein MTP18 [2, 4].

1.3. HUMAN DYNAMIN-RELATED PROTEIN 1 - DRP1

Dynamin-related protein 1 (UniProt number: O00429) is also termed dynamin-like protein (DLP1) or human dynamin IV (HdynIV). The 82 kDa protein is a 736 residue, self-assembling GTPase that mediates mitochondrial fission in humans.

So far, 6 isoforms of DRP1 are described in humans. In literature, isoform 1 (736 amino acids) has been chosen as the `canonical` sequence. The other isoforms lack a part of the flexible linker region and differ in their expression pattern.

The domain architecture comprises a large, amino-terminal GTPase domain, a middle domain, a flexible linker region (also referred to as insert B [1]) and a carboxy-terminal GTPase effector domain (GED). The middle domain and the GED are involved in oligomerization, which leads to enhanced GTPase activity [7]. Therefore, mutations in the middle domain, like A395D which was reported with neonatal death and microcephaly, prevent formation of higher oligomers [11].

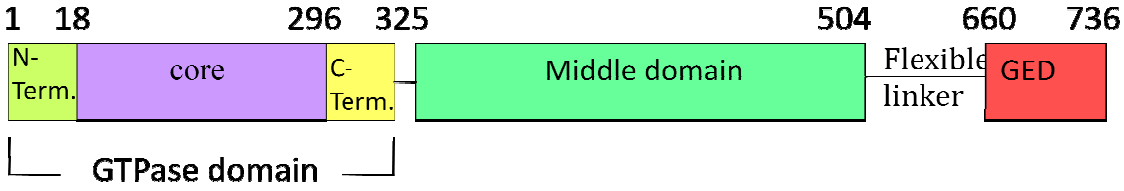


Figure 2. Domain architecture of DRP1.

Several studies have shown that an increase of DRP1 expression, which is predominantly located in the cytosol, does not alter the rate of fission. Post-translational modifications, however, can regulate protein translocation, assembly and GTPase activity [1].

1.3.1. POST-TRANSLATIONAL MODIFICATIONS OF DRP1

PHOSPHORYLATION

Several serine residues of DRP1, namely S616, S637 and S656 can be phosphorylated by a number of protein kinases. This kind of modification has opposite effects: Whereas Benard et al stated that phoshporylation stimulates fission [3], other studies report inhibition of fission [1, 4, 8].

UBIQUITINATION

Usually, polyubiquitination of a protein is a signal for degradation by the proteasome. In this case, it is believed to play a regulatory role in DRP1 assembly [3]. Whether ubiquitination exerts a stimulating or an inhibiting effect on DRP1-mediated fission is not yet clear [1, 4, 8].

SUMOYLATION

SUMO (Small ubiquitin-like modifier), a modulator of protein activity, usually changes the localization of proteins inside the cell or protects against ubiqutin-mediated degradation by binding lysine residues. Again, the effect on DRP1 is controversial [1, 3, 4, 8].

1.4. THE DYNAMIN SUPERFAMILY

The dynamin superfamily is a group of large, self-assembling GTPases that mediates fusion and fission of vesicles and organelles, cytokinesis, pathogen resistance and the formation of the cell plate and the division of chloroplasts in plants [2, 6, 12]. All members of the family are highly conserved across species. Characteristic features include the large GTPase domain, the ability to self-assemble into higher oligomers that form ring- and helix-like structures and the assembly-stimulated GTPase activity.

In contrast to members of the small GTPase family (like ras-like GTPases, α subunits of the hetero-trimeric G-proteins and GTPases involved in protein synthesis), the large GTPase domain of the dynamin superfamily comprises approximately 300 amino acids [6, 13].

Whereas ras-like GTPases need other proteins, the GAPs (GTPase-activating proteins) for GTPase activity, and the GEFs (guanine exchange factors) for exchange of the nucleotide, members of the dynamin superfamily mediate GTPase activation by self-assembly. Due to their relatively low affinity to guanine nucleotides, the fast turn-over rate and the fast dissociation rate of GDP, proteins of the dynamin superfamily do not require nucleotide exchange factors [2, 6, 13].

The GTPase domain contains four GTP-binding motifs, named G1 - G4 (Figure 3.). Each GTPase binds one molecule of GTP:
The G1 motif (consensus sequence: GxxxxGKS) is located in the P-loop and coordinates the γ-phosphate, a conserved threonine residue in G2 is involved in catalysis, the glycine residue in G3 (consensus sequence: DxxG) forms a hydrogen bond with the γ-phosphate of GTP and G4 (consensus sequence: NKxD) coordinates the base and the sugar [6].

	G1	G2	G3	G4
Dynamin1	IAVV**G**GQSA**GKS**SVLENFVG	SGIV**T**RRPLV	LV**D**LP**G**MTKV	VI**TK**L**D**L
DLP1	IVVV**G**TQSS**GKS**SVLESLVG	TGIV**T**RRPLI	LV**D**LP**G**MTKV	VI**TK**L**D**L
MxA	IAVI**G**DQSS**GKS**SVLEALSG	SGIV**T**RCPLV	LI**D**LP**G**ITRV	IL**TK**P**D**L
OPA1	VVVV**G**DQSA**GKT**SVLEMIAQ	GEMM**T**RSPVK	LV**D**LP**G**VINT	VL**TK**V**D**L
Mitofusin	VAFF**G**RTSS**GKS**SVINAMLW	IGHI**T**NCFLS	LV**D**SP**G**TDVT	LN**N**RW**D**A
GBP1	VAIV**G**LYRT**GKS**YLMNKLAG	VQSH**T**KGIWM	LL**D**TE**G**LGDV	VW**T**LR**D**F
Atlastin1	VSVA**G**AFRK**GKS**FLMDFMLR	NEPL**T**GFSWR	LM**D**TQ**G**TFDS	IFLVR**D**W
IIGP1	VAVT**G**ETGS**GKS**SFINTLRG	GAAK**T**GVVEV	FW**D**LP**G**IGST	VR**TK**V**D**S
p21 Ras	LVVV**G**AGGV**GKS**ALTIQLIQ	EYDP**T**IEDSY	IL**D**TA**G**QEEY	VG**NK**C**D**L
EF1α	IVVI**G**HVDS**GKS**TTTGHLIY	ERGI**T**IDISL	II**D**AP**G**HRDF	GV**NK**M**D**S
Consensus	**GxxxxGKS** **T**	**T**	**DxxG**	**NKxD** **T**

Figure 3. GTP-binding motifs G1 – G4; adapted from Praefcke et al, 2004 **(6)**. Identical amino acid residues are shown in red. DLP1 – dynamin-like protein 1, OPA1 – optic atrophy 1, GBP1 – guanylate-binding protein1.

Apart from the GTPase domain, the domain architecture of all members comprises a middle domain and a GTPase effector domain (GED):
The middle domain has been shown to interact with the GED and plays an important role in oligomerization [7, 11, 12, 14, 15].

The backfolding of the GED onto the GTPase-middle domain is also involved in self-assembly and assembly-stimulated GTPase activity [9, 16].
The pleckstrin-homology (PH) domain, as well as the proline-rich domain (PRD) are not present in all dynamin family members. The PH domain mediates binding on phospholipid membranes. The PRD is responsible for interaction with SH3 (Src-homology-3) domains of proteins [6, 16].
The dynamin superfamily can be divided into the dynamins, the dynamin-like proteins (including DRP1, Mx-proteins, OPA1, mitofusin 1 and mitofusin 2), the GBP-related proteins (guanylate-binding proteins) and the Vps1-like proteins (vacuolar protein sorting) [6, 17].

1.4.1. DYNAMINS

The founding member dynamin 1 (UniProt number: Q05193), an 100 kDa protein, catalyzes the fission of clathrin-coated pits from the plasma membrane [16, 18]. Moreover, dynamins are involved in budding of caveolae, in phagocytosis and in cytokinesis [6]. The domain architecture (Figure 4) comprises an amino-terminal GTPase domain, a middle domain, a PH domain, a GED and a PRD [16, 18].
So far, 5 isoforms are known in humans. Dynamin has been shown to exist as a tetramer within cells [12, 19].

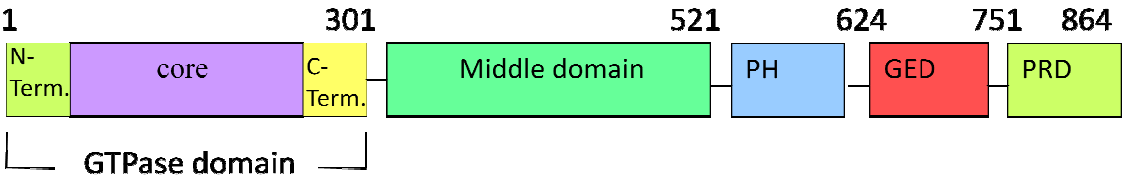


Figure 4. Domain architecture of human dynamin 1. PH – pleckstrin homology, GED – GTPase effector domain, PRD – proline rich domain.

1.4.2. DYNAMIN-LIKE PROTEINS

All dynamin-like proteins (Dlp´s) lack the PRD. They fulfill a variety of important functions in cells [6].
Human OPA1 (optic atrophy 1) and Mgm1 (mitochondrial genome maintenance 1) from S. cerevisiae mediate fusion of the inner membrane of mitochondria. They contain a mitochondrial targeting - and a transmembrane sequence.
Fusion of the outer mitochondrial membrane involves Mfn´s (mitofusins) in humans and Fzo1 (fuzzy onions 1) in yeast [6, 20].
Mitochondrial fission is catalyzed by DRP1 (dynamin-related protein 1) and Dnm1 (dynamin-like protein 1) in humans and yeast, respectively.
Mx-like proteins (human MxA and MxB) are expressed upon type I interferon production and protect against viruses. GBPs/atlastins mediate protection against intracellular pathogens.

1.5. THE STRUCTURE OF DYNAMIN AND OTHER MEMBERS OF THE DYNAMIN SUPERFAMILY

In 2001, the structure of nucleotide-free and GDP-bound form of the GTPase domain of dynamin A from Dictyostelium discoideum was solved (PDB ID 1JWY and 1JX2) [21] and in 2005, Reubold et al managed to solve the crystal structure of the rat dynamin GTPase domain (PDB ID 2AKA) [22]. In the last few years, the structures of several members of the dynamin superfamily, like the stalk of MxA (PDB ID 3LJB) [23], the bacterial dynamin-like protein (PDB ID 2J68) [24] and the guanylate-binding protein1 (PDB ID 1DG3) [25], have been solved.

In 2010, Chappie et al crystallized a part of human dynamin 1 (monomeric GTPase domain-GED fusion protein, PDB ID 2X2E) [16]. By adding GDP, sodium flouride and aluminium chloride, a dimeric transition state mimic was formed.
Chappie et al proposed a model for dynamin assembly, which explains the enhanced GTPase activity of dynamin upon oligomerization:

In full length dynamin, the C-terminal part of the GED interacts with the GTPase domain in trans to form a dimer. Further on, two dimers assemble via their stalk to form a tetramer. Between tetramers, G domain dimerization leads to formation of higher oligomers [16, 26].
Dimerization of the GTPase domains induces a conformational change in the active site that brings the different parts of the catalytic machinery together and leads to assembly-stimulated GTPase activity [16]. As a result, the catalytic water molecule, that mediates a nucleophilic attack on the γ-phosphate of GTP, is positioned at the correct site. The developing negative charge is compensated by a sodium ion and a magnesium ion and flexible switch regions are stabilized.
Recently, the crystal structures of rat dynamin 1 [27] and human dynamin 1 [18], both lacking the PRD, have been solved.

1.5.1. CRYSTAL STRUCTURE OF HUMAN DYNAMIN

The GTPase domain of human dynamin consists of a curved beta-sheet of six strands, surrounded by five α-helices [6, 18]. The BSE (bundle signaling element) is built up of three α-helices from the amino-terminal GTPase domain, the carboxy-terminal GTPase domain and the carboxy-terminal part of the GED.
The bundle signaling element is connected to the stalk – a bundle of four anti-parallel helices formed by the middle domain and the amino-terminal GED [18, 27].

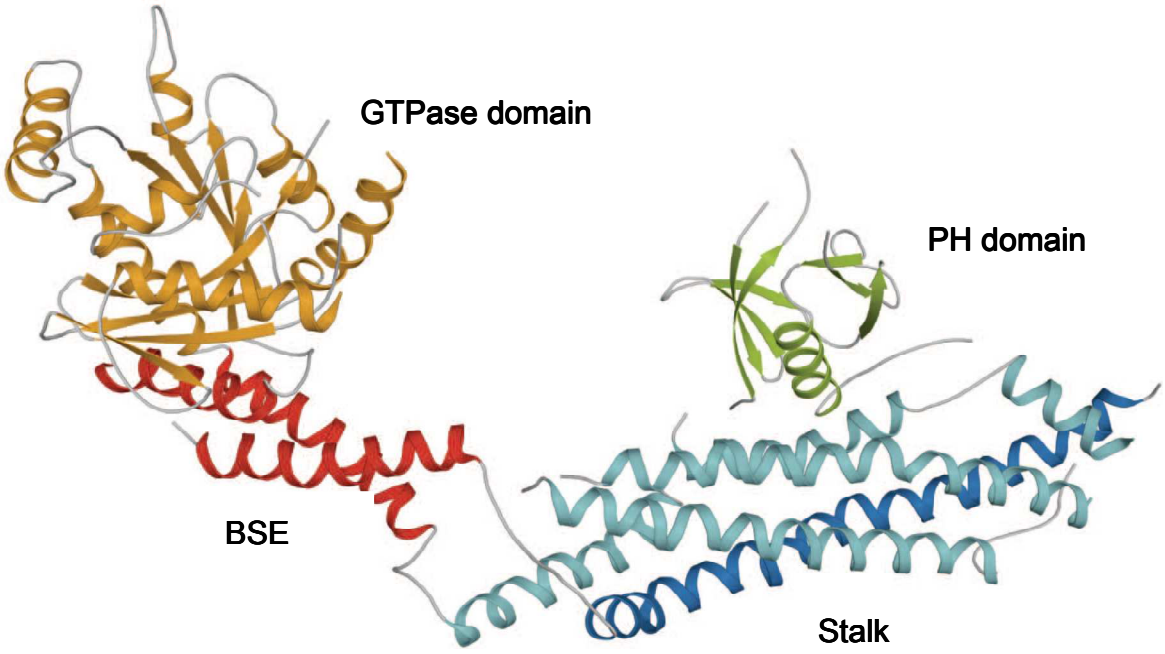


Figure 5. Structure of nucleotide free human dynamin 1, adapted from Faelber et al [18].

In 2011, Faelber et al managed to solve the crystal structure of human, nucleotide-free dynamin that lacks the PRD (PDB ID 3SNH) [18]:

Oligomerization is mediated through assembly of the stalks of neighbouring molecules, which are arranged in a criss-cross fashion, building a linear filament. Within the stalk, three conserved interfaces are involved in the assembly of higher order structures.

Upon GTP binding, rearrangements in the stalk induce a bent. In the helix, the GTPase domains of neighbouring molecules come together and GTPase activity is enhanced [18].

1.5.2. CRYSTAL STRUCTURE OF RAT DYNAMIN

Also in 2011, Ford et al solved the crystal structure of nucleotide-free rat dynamin 1 lacking the PRD (PDB ID 3ZVR) [27]. Like in human dynamin, a linear filament is built up by dimers that assemble via three interfaces in the stalk. Upon GTP binding and assembly, helix formation – the linear filament gets positioned into rings – is mediated by a GTPase-GTPase interface. Furthermore, Ford et al states that the PH domain might link the dynamin assembly to membrane interactions by regulating access to one of these interfaces that shifts during helix formation [27].

Most recently, the crystal structure of the GTPase domain and the GED of dynamin-related protein 1 A from A. thaliana was solved by Chen et al [28].

1.6. DYNAMIN-LIKE PROTEINS AND THEIR ROLE IN DISEASE

The process and proper function of mitochondrial dynamics is particularly important in cells of the nervous system, amongst others due to their high energy demand. Thus, defects in mitochondrial fission or fusion lead to various neurodegenerative disorders [4, 8]:
Mutations in the gene encoding mitofusin 2 cause Charcot-Marie-Tooth neuropathy. OPA1 mutations are associated with dominant optic atrophy type 1 (DOA) [3].
A mutation in the middle domain of DRP1, namely A395D – a highly conserved residue, has been reported to cause microcephaly and abnormal brain development which leads to neonatal death. This mutation inhibits higher order assembly and probably translocation of DRP1 to the OM. Consistently, a decrease in GTPase activity was reported, which in turn leads to elongated mitochondria [11].
Alzheimer´s disease (AD) and Parkinson´s disease (PD) are associated with mitochondrial dysfunction that results in inhibition of the respiratory system and oxidative damage due to an increased production of reactive oxygen species (ROS) [8, 29].

In Huntington´s disease, the gene encoding huntingtin (Htt) is mutated. The function of wildtype Htt within cells is not known. The insertion of multiple CAG repeats leads to the attachment of polyglutamine to the N-terminus of the protein [29]. The number of glutamine residues (6 - 35 in the wildtype protein) has been shown to correlate with the severity of the disease. Recently, Song et al showed that mutant huntingtin – in comparison to the wildtype protein - exhibits a stronger interaction with DRP1, thereby increasing its activity. This leads to fragmentation of cristae and mitochondria. Later on, Htt aggregation and neuronal cell death is observed. Thus, DRP1 might be a new drug target in treatment of Huntington´s disease [30] and therefore, its crystal structure is of great interest and high clinical relevance.

2. MATERIAL AND METHODS

2.1. CONSTRUCTS

Constructs listed below have been optimized for crystallizing full length or at least parts of DRP1 and DNM1. Different experimental approaches include the removal of variable regions and introduction of mutations that inhibit higher order assembly, since both might impede crystallization.

2.1.1. DNM1 (YEAST)

DNM1 (Dynamin-related protein 1) was a gift from the laboratory of Jodi Nunnari.

DNM1 constructs

	Construct name	Construct design	Aa	MW (kDa)	Vector	His (6)-tag
Wildtype	**E 59***	M1 – L765	765 aa	86	pHIS-P2	N-term.
	E 60*	M1 - L 782	782 aa	88,1	pET-21	C-term.
G385D Mutation full length	**E 61**	M1 - L765	765 aa	86,1	pHIS-P2	N-term.
	E 62*	M1 - L782	782 aa	88,2	pET-21	C-term.

Table 1. List of DNM1 constructs E 59, E 60, E 61 and E 62 with corresponding number of amino acids (aa) and molecular weight. Constructs labeled with a star were cloned by Mag. Klinglmayr.

2.1.2. DRP1 (HUMAN)

DRP1 (Dynamin-related protein 1, Isoform 1) was a gift from the laboratory of Ella Bossy-Wetzel.

DRP1 constructs

	Construct name	Construct design	Aa	MW (kDa)	Vector	His (6)-tag
GTPase domain-GED fusion constructs	**E 63***	M1-D328 fused with K711- W736	369	40,9	pET-21	C-term.
	E 64	M1-D328 fused with K711- W736	386	43	pHIS-P2	N-term.
	E 65	M1-D328 fused with K635- W736	437	49,2	pET-21	C-term.
	E 66*	M1-D328 fused with K635- W736	454	51,3	pHIS-P2	N-term.
Mutation constructs	**E 67**	M1- W 736: A395D	736	83	pET-21	C-term.
	E 68	M1- W 736: G350D	736	83	pET-21	C-term.
	E 69	M1- W 736: G363D	736	83	pET-21	C-term
GTPase domain constructs	**E 70***	M1 - S321	348	39,1	pHIS-P2	N-term.
	E 71*	M1 - G323	350	39,4	pHIS-P2	N-term.
	E 72*	M1 - D327	354	39,8	pHIS-P2	N-term.

Table 2. List of DRP1 constructs E 63 – E 72 with corresponding number of amino acids (aa) and molecular weight. Constructs labeled with a star were cloned by Mag. Klinglmayr.

Note: In the following, “GTPase domain-GED fusion protein” refers to the protein encoded by construct E 63.

2.2. VECTORS

2.2.1. PET-21

The pET-21 vector was obtained from Novagen: It is 5450 bp long and encodes a C-terminal His-tag under the control of a T7 promoter and Ampicillin for selection.

2.2.2. pHIS-PARALLEL 2

The multiple cloning site of pHIS-Parallel 1 was modified in a way that cloning with NdeI and XhoI leads to the attachment of a N-terminal 6 x His tag, which can be cleaved by the rTEV protease. The modified vector pHIS-Parallel 2 used in this study is ~ 5500 bp long and encodes Ampicillin for selection.

2.3. CLONING

As already mentioned, cloning of DNM1 constructs E 59, E 60 and E 62 as well as DRP1 constructs E 63, E 66 and E 70 – E 72 was performed by Mag. Klinglmayr.
DNM1 G385D mutation construct E 61 was subcloned into vector pHIS-P2 by performing PCR. The DNM1 G385D DNA sequence in another vector was used as a template.

2.3.1. PCR

PCR was performed using a Thermal Cycler (Eppendorf). Primer sequences are listed in the Appendix.

PCR reaction setup (E 61):

DNM1 G385D DNA	0,2 µL
2 x Mastermix*	12,5 µL
DNM1 primer forward (100 µM)	0,25 µL
DNM1 primer reverse (100 µM)	0,25 µL
dd H_2O	11,8 µL
	25 µL

Table 3. PCR reaction setup for construct E 61.* iProof High-Fidelity Master Mix (Bio-Rad) contains reaction buffer, 0,04 u/µL iProof polymerase and 400 µM dNTPs.

PCR program:

→ Initial denaturation: 1 x 98°C 30´´
→ Denaturation: 30 x 98°C 10´´
→ Annealing: 60°C 20´´
→ Extension: 72°C 1´10´´
→ Final extension: 1 x 72°C 7´
→ Cool down to: 4°C.

2.3.2. OVERLAP PCR

Cloning of DRP1 constructs E 64, E 65, E 67 – E 69 was carried out by performing overlap PCR. Primer design was performed with PrimerX (http://www.bioinformatics.org/primerx/). Sequences are listed in the Appendix.

PCR 1 – 5´part		PCR 1 – 3´part	
DRP1 fl DNA	0,2 µL	DRP1 fl DNA	0,2 µL
2 x Mastermix*	12,5 µL	2 x Mastermix*	12,5 µL
DRP1 primer forward (100 µM)	0,25 µL	DRP1 primer reverse (100 µM)	0,25 µL
Fusion/Mutation primer reverse (100 µM)	0,25 µL	Fusion/Mutation primer forward (100 µM)	0,25 µL
dd H_2O	11,8 µL	dd H_2O	11,8 µL
	25 µL		25 µL

Table 4. PCR 1 reaction setup.* iProof High-Fidelity Master Mix (Bio-Rad) contains reaction buffer, 0,04 u/µL iProof polymerase and 400 µM dNTPs.

PCR program:

→ Initial denaturation: 1 x 98°C 30´´
→ Denaturation: 30 x 98°C 10´´
→ Annealing: 60°C 20´´
→ Extension: 72°C 1´
→ Final extension: 1 x 72°C 7´
→ Cool down to: 4°C.

PCR products were loaded onto a preparative 1% agarose gel. The gel slices with the bands were excised and centrifuged for 10 minutes at RT in a table top centrifuge (Biofuge pico, Heraeus). The supernatant was used as template for PCR 2:

PCR 2 - elongation	
5´part of PCR1	1 µL
3´part of PCR1	1 µL
2 x Mastermix*	25 µL
dd H_2O	22 µL
	49 µL

Table 5. PCR 2 reaction setup. * iProof High-Fidelity Master Mix (Bio-Rad) contains reaction buffer, 0,04 u/µL iProof polymerase and 400 µM dNTPs.

PCR program:

→ Initial denaturation: 1 x 98°C 30´´
→ Denaturation: 7 x 98°C 10´´
→ Annealing: 50°C 25´´
→ Extension: 72°C 1´10´´
→ Final extension: 1 x 72°C 7´
→ Cool down to: 4°C.

PCR 3

0,5 µL of each DRP1 primer (100 µM) were added to the PCR product of PCR 2.

PCR program:

→ Initial denaturation: 1 x 98°C 30´´
→ Denaturation: 35 x 98°C 10´´
→ Annealing: 62°C 25´´
→ Extension: 72°C 1´30´´
→ Final extension: 1 x 72°C 7´
→ Cool down to: 4°C.

Again, PCR products were loaded onto a preparative 1% agarose gel. The bands were excised and DNA was extracted using Wizard® SV Gel and PCR Clean-Up System (Promega).

2.3.3. DIGESTION

Digestion of PCR products and vectors was performed with 2 µL of Fast digest restriction enzymes NdeI and XhoI (Fermentas Life Sciences, 1u/µL) for 1 hour at 37°C.

Digestion

DNA	24 µL
NdeI (1u/µL)	2 µL
XhoI (1u/µL)	2 µL
10 x FastDigest buffer*	4 µL
dd H_2O	8 µL
	40 µL

Table 6. Reaction setup for digestion. *10 x FastDigest buffer was provided by Fermentas Life Sciences together with the restriction enzymes.

After digestion, heat inactivation of the restriction enzymes was performed at 80°C for 10 minutes. In order to prevent re-ligation of digested vector, dephosphorylation was carried out for 10 minutes at 37°C by using 2 µL FastAP Thermosensitive Alkaline Phosphatase (Fermentas Life Sciences, 1u/µL). Dephosphorylated vectors were loaded onto an agarose gel and gel slices were excised. Vectors and inserts were cleaned up using Wizard SV Gel and PCR Clean-Up System (Promega).

2.3.4. LIGATION

Ligation was performed at RT for at least 30 minutes using 0,5 µLT4 DNA ligase (Fermentas Life Sciences, 5 u/µL).

Ligation	
Ligase (5u/µL)	0,5 µL
10 x T4 DNA ligase buffer *	1 µL
Vector	x µL
Insert	x µL
dd H_2O	x µL
	10 µL

Table 7. Reaction setup for ligation. * T4 DNA Ligase buffer was provided by Fermentas Life Sciences together with the T4 DNA ligase and contains 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT and 5 mM ATP.

The vector insert ratios were calculated as follows, the molar ratio of insert/vector used was 3:1.

$$\frac{\text{(ng vector) x (size of insert in kB)}}{\text{(size of vector in kB)}} \times \text{molar ratio of (insert/vector)} = \text{ng insert}$$

2.3.5. TRANSFORMATION

After ligation, plasmids were transformed into chemically competent E.coli Top 10 (Invitrogen) by heat shock:
50 µL bacteria were thawed on ice. 5 µL plasmid were added. After tapping, samples were incubated on ice for 10 minutes, followed by an incubation at 42°C for 1 minute. To recover cells, 500 µL LB-medium (Luria Broth medium) were added and incubated for 45 minutes at 37°C while shaking. Bacteria were plated on LB Amp plates.

2.3.6. COLONY PCR

Colony PCR was performed by using 0,25 µL Dream Taq Green DNA Polymerase (Fermentas Life Sciences, 5 u/µL) to control the efficiency of transformation. Single colonies were picked and used as templates:

Colony PCR	
Bacterial cells	
dNTP Mix° (0,2 mM)	0,65 µL
T 7 primer forward (100 µM)	0,25 µL
T7 primer reverse (100 µM)	0,25 µL
10 x Dream Taq buffer*	2,5 µL
Dream Taq Polymerase (5 u/µL)	0,25 µL
dd H_2O	21,1 µL
	25 µL

Table 8. Reaction setup for colony PCR. ° 0,2 mM dNTP Mix was supplied with Dream Taq Polymerase from Fermentas Life Sciences and used at a final concentration of 0,5 mM. *10 x Dream Taq buffer was provided by Fermentas Life Sciences together with the Dream Taq Polymerase and contains KCl, $(NH_4)_2$ SO_2 and 20 mM $MgCl_2$.

PCR program:

- → Initial denaturation: 1 x 95°C 3´
- → Denaturation: 35 x 95°C 30´´
- → Annealing: 50°C 30´´
- → Extension: 72°C 2´30´´
- → Final extension: 1 x 72°C 7´
- → Cool down to: 4°C.

Plasmid preparations were performed using a GeneJET Plasmid Miniprep Kit (Fermentas Life Sciences) and sent to Eurofins MWG Operon for sequencing.

2.3.7. AGAROSE GEL ELECTROPHORESIS

Visualization of DNA was performed with Gel Red (Biotium Inc.). Gels were run at 100 V for 40 minutes and visualization was carried out by Ultraviolet Transilluminator Gel Doc-It Imaging system (BioImaging Systems).

2.4. PROTEIN EXPRESSION

Sequenced DNM1 and DRP1 constructs were transformed into chemically competent E. coli BL-21 DE 3 (Invitrogen) cells by heat shock: 50 µL bacteria were thawed on ice. 5 µL plasmid were added. After tapping, samples were incubated on ice for 10 minutes, followed by an incubation at 42°C for 1 minute.To recover cells, 500 µL LB-medium (Luria Broth medium) were added and incubated for 45 minutes at 37°C while shaking. Bacteria were plated on LB plates supplemented with Ampicillin (50 µg/mL) and incubated at 37°C overnight.

Colonies were resuspended from LB plates, transferred into 1 x 600 mL LB Amp medium (50 µg/mL Ampicillin) and grown for 4h at 37°C (250 rpm, Rocker 25, Labnet International Inc.) until OD_{600} ~ 0.8.

Protein expression was induced by addition of 0,45 mM IPTG and performed at 25°C overnight. Cells were harvested by centrifugation at 4500 rpm for 15 minutes (Sorvall RC 6. SLC-4000, Thermo Electron Corporation), pellets were resuspended in 1/20 volume of Lysis buffer (see Appendix) and frozen at – 20°C overnight.
For large scale expressions up to 12 flasks á 600 mL were combined.

2.5. PROTEIN PURIFICATION

2.5.1. CELL LYSIS

After thawing of resuspended pellets, 150 µL protease inhibitor PMSF (see Appendix) at a final concentration of 1 mM were added to each pellet (obtained from 1 x 600 mL bacterial culture) and cells were disrupted by sonification (HD 2070 Bandelin Sonopuls SH 706) for 2 x 3 minutes at full power. Next, DNA was digested for 45 minutes on ice using 20 µL RNase-free DNase I (Fermentas Life Sciences, 1 u/µL). Lysate was cleared by centrifugation at 18.000 rpm on 4 °C for 20 minutes (Sorvall RC 6. SS-34, Thermo Electron Corporation) and supernatant was subjected to immobilized metal affinity chromatography.

2.5.2. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

The supernatant was loaded twice by gravity flow on a 20 mL column (pre-equilibrated with Equilibration buffer, see Appendix) packed with Ni^{2+} Sepharose Fast Flow resin (GE Healthcare). Bound protein was washed with 2 column volumes of Equilibration buffer (see Appendix) and Wash buffer (see Appendix), respectively. The protein was eluted with 3 mL Elution buffer (see Appendix), subjected to SDS page analysis and to further purification using SEC.

2.5.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein samples taken from the supernatant, the flow-through, the pellet, the elutions and the beads were mixed with an equal amount of SDS loading buffer (see Appendix). Samples were heated at 95°C for 5 minutes. 10 µL of each sample and 2 µL of prestained protein ladder (Fermentas Life Sciences) were loaded onto a 15 % SDS- Polyacrylamide gel (preparation see Appendix). Gels were run at 225 V for 45 minutes.

SDS gels were stained with Coomassie present in the Staining solution (see Appendix) for 10 minutes at RT after a short heating period (approximately 10 seconds) in the microwave. After removing Staining solution, the gel was put into Destaining solution (see Appendix) overnight and stored in dd H_2O.

2.5.4. BUFFER EXCHANGE

Buffer exchange of protein elutions from IMAC was carried out using PD-10 or G-25 desalting columns (GE Healthcare).

2.5.5. SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Elutions from IMAC were further purified by size exclusion chromatography at 4°C using a Superdex 75 10/300 GL column (GE Healthcare). Per run, 600 µL protein sample were injected (flow rate: 0,4 mL/min, emptying of 1 mL loop: 1,8 mL). Unless otherwise stated, SEC analysis was performed in SEC buffer (see Appendix).

Calibration of the size exclusion column: A mix of proteins (Table 9) was loaded onto the S-75 column to determine a molecular weight calibration curve. The standard curve was used to calculate the elution volume of the monomeric GTPase domain-GED fusion protein:

Protein	Molecular weight	Retention volume
C - Conalbumin	75 kDa	9,81 mL
O – Ovalbumin	43 kDa	10,48 mL
CA – Carboanhydrase	29 kDa	11,87 mL
R - Ribonuclease	13,7 kDa	13,61 mL

Table 9. Protein mix loaded onto the Superdex 75 column.

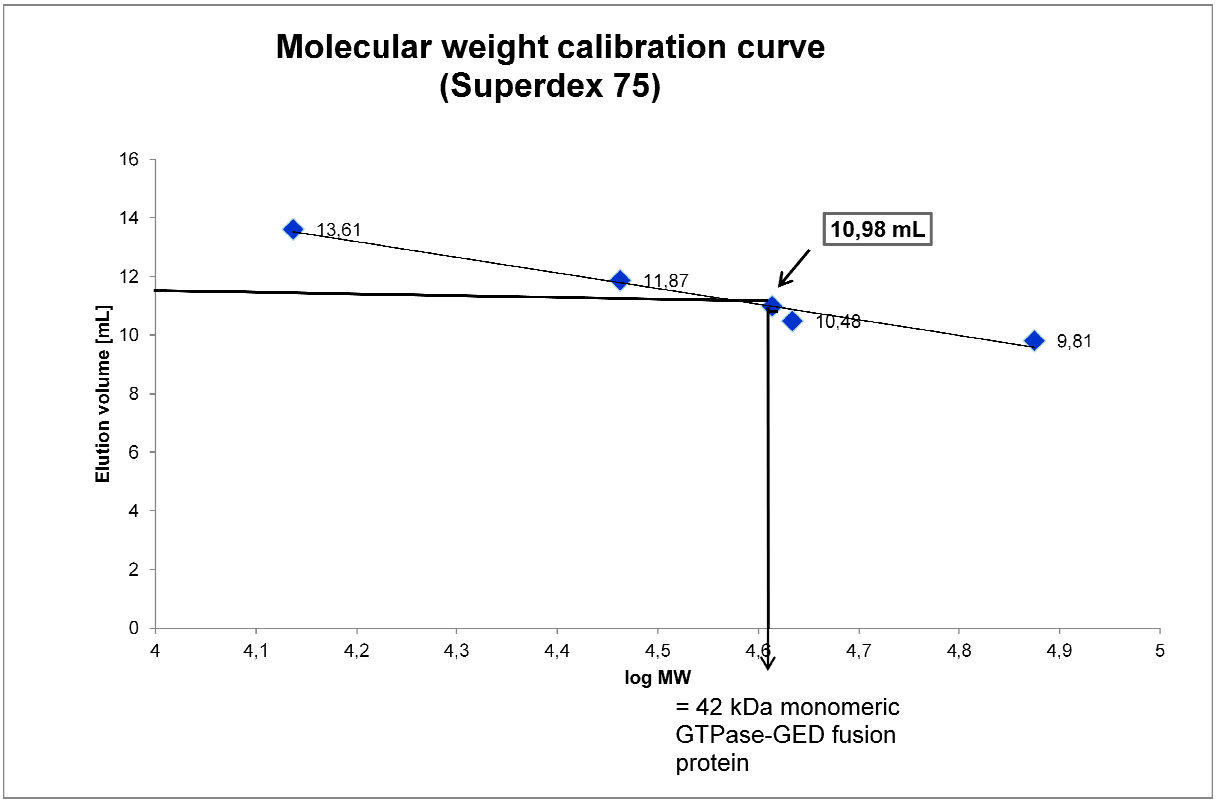


Figure 6. Calibration curve of S-75 column. Elution volume of 10,98 mL corresponds to the monomeric form of the GTPase-GED fusion protein. MW - molecular weight.

For further experiments, FPLC fractions containing protein were pooled and concentrated up to 10 mg/mL by Amicon Ultra-4 centrifugal filter devices (Molecular weight cut off: 10.000; Millipore). Protein concentration was determined by UV_{280} and aliquots of 30 µL were prepared. Purified protein was immediately put into the freezer where it was stored at -80°C.

2.6. DYNAMIC LIGHT SCATTERING (DLS)

In order to check the homogeneity and the oligomerization state of the protein, dynamic light scattering was performed:
Purified protein was diluted to 0,25 mg/mL and 80 µL of the sample were put into a disposable, single sealed UVette (50 – 2000 µL, Eppendorf) and measured at 20 °C (10 acquisitions á five seconds) by using the DynaProNanoStar Instrument (Wyatt). Scattered intensity and radius of the measured particle were plotted in a size distribution histogram and percent polydispersity was calculated.

2.7. PROTEIN THERMAL SHIFT ASSAY (THERMOFLUOR ASSAY)

The Thermofluor assay was performed in order to examine protein stability in different buffers:

$$c\ (g/mol) = \frac{c\ (g/L)}{M\ (g/mol)}$$

c...concentration
M...molarity

The protein sample was diluted in a buffer containing 20 mM HEPES and 100 mM NaCl , pH 7.4, to reach an end concentration between 2 and 10 µM in 4 µL.
Non-specific dye S. Orange (SYPRO Orange protein gel stain) was supplied by Invitrogen as a 5000x stock and was diluted to 4,5x in a volume of 6 µL. Buffers were pipetted in a 96 well plate (10 µL/well) and 4 µL protein, 6 µL S. Orange and 10 µl of 2x S. Orange buffer were added.
Absorbance at 568 nm was plotted against temperature in a melt curve diagram. Calculated melting temperatures (Tm) from different conditions were compared. A list of all tested buffers can be found in the Appendix.

2.8. CONTINUOUS REGENERATIVE COUPLED GTPASE ASSAY

GTPase activity of DRP1 (turnover/min) was determined with the continuous, regenerative coupled GTPase assay described by Ingerman and Nunnari in 2005 [31].
First, a buffer exchange of the protein into the GTPase Assay buffer (see Appendix) was performed. In order to obtain a final concentration of 0,1 mg/mL, a 1,4 mg/mL stock of DRP1 was prepared. Likewise, a GTP stock (10 mM) was prepared and serially diluted in 25 mM HEPES and 25 mM PIPES.

A mastermix containing 10 µL 20x Reaction buffer (see Appendix), 12 µL 10mM NADH, 12 µL 2,3 M NaCl, 126 µL GTPase Assay buffer (see Appendix), 5 µL of a pyruvate kinase and lactate dehydrogenase mix (Sigma-Aldric Co.) and 15 µL protein was prepared and aliquoted (180 µL/well) into a 96 well plate. 20 µL of the GTP stocks were added leading to a final concentration of 0, 10, 25, 50, 100, 250, 500 and 1000 µM GTP. Absorbance at 340 nm (120 cycles á 20 seconds at 30°C, Infinite M 200 Tecan) was plotted against time. For final analysis, GTP concentration and corresponding GTPase activity (turnover/min) were plotted against each other.

2.9. CRYSTALLIZATION

The GTPase domain-GED fusion protein used for crystallization was monomeric and in SEC buffer (see Appendix). To determine the optimal protein concentration for crystallization, a PCT- Pre-Crystallization test (HR-140, Hampton research) was performed.
Initial crystallization screening was performed using the following commercially available crystallization screens: Hampton Index screen HR2-144 (Hampton research), JCSG-plus screen MD1-37 (Molecular dimensions) and PEG suite.
0,4 µL protein were mixed with 0,4 µL reservoir solution by using a Hydra II Plus One (Matrix Ltd.) liquid-handling system and equilibrated against 50 µL reservoir solution. Plates were stored at 4°C/20°C and checked for crystals routinely.

Initial hits were refined manually using 96- well intelli plates as well as 24- well plates, performing hanging - and sitting-drop vapor diffusion techniques. For fine screening, protein and precipitant concentration, and pH were modified. Moreover, additive screens, microseeding and streakseeding were performed [32, 33].

2.10. X-RAY DIFFRACTION ANALYSIS

Suitable crystals were flash-frozen in liquid nitrogen. Initial diffraction measurements were performed at an in house X-ray diffractometer using a MAR345 image plate system (MAR Research, Hamburg) on a Bruker Microstar rotating anode system at 100 K.

An X-ray diffraction data set was collected on beamline ID14-4 at the ESRF Grenoble. The beamline was equipped with a Q315r ADSC CCD detector and data collection was performed using a wavelength of 1 Å and a crystal-to-detector distance of 400 mm. 180 images were collected at 100 K with a 1.0° oscillation range.

Diffraction images were processed by iMOSFLM (Battye et al., 2011) and SCALA from the CCP4 program suite (Winn et al., 2011).

3. RESULTS

3.1. CONSTRUCT DESIGN

Following constructs have been designed:

	Construct name	Construct design	Cloning	Express-ion	Purifi-cation	Crystallization/ GTPase assay
DNM1 constructs						
Wildtype	**E 59***	M1 – L765	✓			
	E 60*	M1 - L 782	✓			
Full length mutations G385D	**E 61**	M1 - L765: G385D	✓	✓		
	E 62*	M1 - L782: G385D	✓			
DRP1 constructs						
GTPase domain-GED fusions	**E 63***	M1 -D328 fused with 8xGS linker and K711 - W736	✓	✓	✓	✓
	E 64		✓	✓		
	E 65	M1 -D328 fused with flexible linker and K635 - W736	✓	✓		
	E 66*		✓			
Full length mutations	**E 67**	M1- W 736: A395D	✓	✓		
	E 68	M1- W 736: G350D	✓	✓		
	E 69	M1- W 736: G363D	✓	✓		
GTPase domains	**E 70***	M1 - S321	✓	✓	✓	
	E 71*	M1 - G323	✓	✓	✓	
	E 72*	M1 - D327	✓	✓	✓	

Table 10. Overview of DNM1 and DRP1 constructs. Constructs labeled with a star were cloned by Mag. Klinglmayr.

3.1.1. DNM1 (YEAST)

DNM1 (Dynamin-related protein 1) is the yeast ortholog of DRP1 and has a molecular weight of 85 kDa. It consists of a N-terminal GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED). DNM1 full length and a G385D mutation have been cloned into pHIS-Parallel2 and pET-21, attaching a N- and a C-terminal His-tag to the construct, respectively. The G385D substitution is located in the middle domain and was described to form a stable dimer [15].

3.1.2. DRP1 (HUMAN)

DRP1 (Dynamin-related protein 1) is a 82,9 kDa protein. It consists of a N-terminal GTPase domain and a middle domain, which is linked via a flexible linker region to the C-terminal GTPase effector domain.

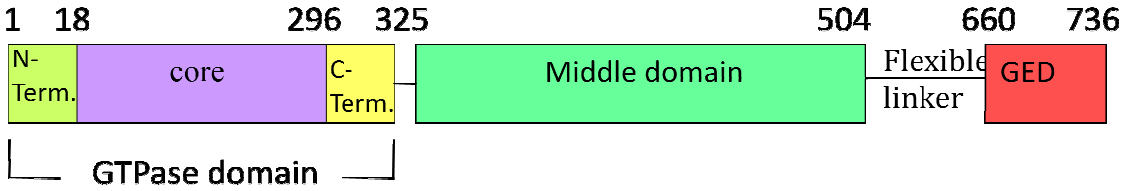


Figure 7. Domain architecture of DRP1. GED – GTPase effector domain.

Fusion constructs E 63 – E 66 have been cloned into the vector pHIS-Parallel2 and pET-21, attaching a N- and a C-terminal His-tag to the construct, respectively. E 63 and E 64 are 1422 bp long fusions of the GTPase domain and the C-terminal part of the GED, connected with a glycine-serine linker. Since these proteins lack the middle domain, they cannot form higher oligomers. Construct design was performed according to the human dynamin 1-derived minimal GTPase domain-GED fusion protein, crystallized by Chappie et al in 2010 [16].

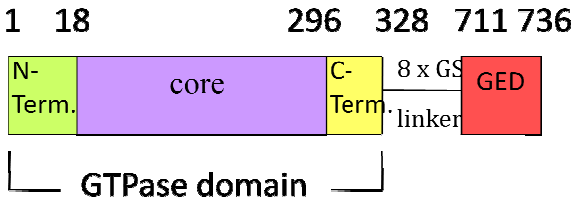


Figure 8. Domain architecture of constructs E 63/E 64.The N-terminal/ C-terminal His-tag is not shown. GS linker – Glycine-serine linker, GED – GTPase effector domain.

E 65 and E 66 are similar fusion constructs, containing an additional part of the flexible linker region and the whole GTPase effector domain.

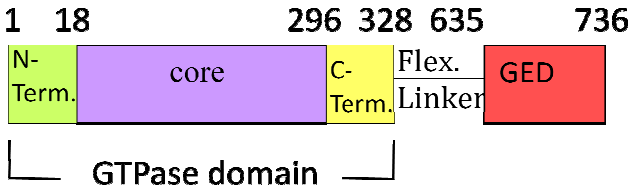


Figure 9. Domain architecture of constructs E 65/ E 66. The N-terminal/ C-terminal His-tag is not shown. Flex. Linker – Flexible linker region. GED – GTPase effector domain.

Mutation constructs E 67 – E 69 are full length DRP1 constructs carrying the mutations A395D, G350D - which corresponds to the G385D substitution in yeast - and G363D.

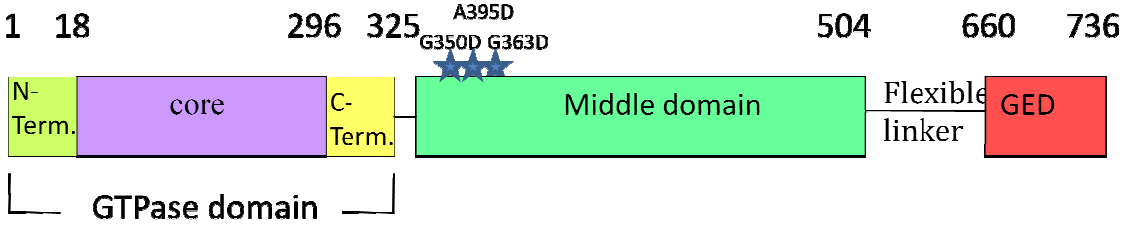


Figure 10. Domain architecture of constructs E 67, E 68 and E 69. GED – GTPase effector domain. Blue stars indicate sites of mutation.

All of these mutated amino acids are located in the middle domain, highly conserved across different species (Figure 11) and have been described to inhibit higher order assembly and mitochondrial fission: G350D - and G363D mutants are able to form tetramers, but not higher oligomers. A395D mutants exist mainly in a dimeric state [11].

```
H. sap.  EPVDDKSATLLQLITKFATEYCNTIEGTAKYIETSELCGGARICYIFHETFGRTLESVDP 383
M. musc. EPVDDKSATLLQLITKFATEYCNTIEGTAKYIETSELCGGARICYIFHETFGRTLESVDP 389
C. ele.  EPVEDKNRTLLQIITRFATAYTSTIEGTARNIETTELCGGARICYIFHDTFGRSLESVNP 385
S. cere. TTNESRASLVLQLMNKFSTNFISSIDGTSSDINTKELCGGARIYYIYNNVFGNSLKSIDP 418
          . :.:   :**:::*:* : .:*:**:  *:*.******** **:::.**.:*:*::*

H. sap.  LGGLNTIDILTAIRNATGPRPALFVPEVSFELLVKRQIKRLEEPSLRCVELVHEEMQRII 443
M. musc. LGGLNTIDILTAIRNATGPRPALFVPEVSFELLVKRQIKRLEEPSLRCVELVHEEMQRII 449
C. ele.  LENLTQLDILTAIRNATGPRPALFVPEVSFELLVKRQIQRLEEPSLRCVELVHEEMQRMV 445
S. cere. TSNLSVLDVRTAIRNSTGPRPTLFVPELAFDLLVKPQIKLLLEPSQRCVELVYEELMKIC 478
           .*. :*: *****:*****:*****::*:**** **: * *** ******:**: ::
```

Figure 11. Section of a multiple sequence alignment (obtained from ClustalW2) of DRP1 and its orthologs in different species showing conserved amino acids in the middle domain. H. sap.refers to Dynamin related protein 1 from H. sapiens (UniProt-number: O00429), M. musc. refers to Dynamin 1-like protein from M. musculus (UniProt-number: Q8K1M6), C. ele. refers to Dynamin related protein 1 from C. elegans (UniProt-number Q9U4L0) and S. cere. refers to Dynamin-related protein 1 from S. cerevisiae (UniProt-number: P54861).

Finally, already existing **GTPase domain constructs** in the lab of different length were subcloned into pHIS-Parallel 2, in order to attach a N-terminal His tag to the protein to increase crystallization probability. These constructs are termed **E 70, E 71 and E 72**.

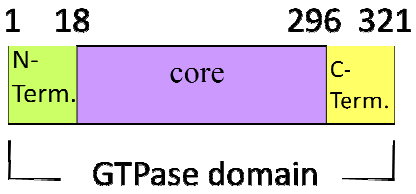


Figure 12. Domain architecture of constructs E 70, E 71 and E 72. The N-terminal His-tag is not shown.

3.2. BIOINFORMATIC ANALYSIS OF THE DRP1 GTPASE DOMAIN-GED FUSION PROTEIN

The DNA sequence of DRP1 was obtained from **NCBI** (NCBI Reference Sequence: NP_036192.2, UniProt number: O00429, listed in the Appendix).

The amino acid sequence of the GTPase domain-GED fusion protein:

```
        10         20         30         40         50         60
MEALIPVINK LQDVFNTVGA DIIQLPQIVV VGTQSSGKSS VLESLVGRDL LPRGTGIVTR

        70         80         90        100        110        120
RPLILQLVHV SQEDKRKTTG EENGVEAEEW GKFLHTKNKL YTDFDEIRQE IENETERISG

       130        140        150        160        170        180
NNKGVSPEPI HLKIFSPNVV NLTLVDLPGM TKVPVGDQPK DIELQIRELI LRFISNPNSI

       190        200        210        220        230        240
ILAVTAANTD MATSEALKIS REVDPDGRRT LAVITKLDLM DAGTDAMDVL MGRVIPVKLG

       250        260        270        280        290        300
IIGVVNRSQL DINNKKSVTD SIRDEYAFLQ KKYPSLANRN GTKYLARTLN RLLMHHIRDC

       310        320        330        340        350        360
LPELKTRINV LAAQYQSLLN SYGEPVDGSG SGSGSKEAAD MLKALQGASQ IIAEIRETHL

WLEHHHHHH
```

Figure 13. Amino acid sequence of the GTPase domain-GED fusion protein (D328 fused with K711) showing the N-term. GTPase in green, the core GTPase in purple, the C-term. GTPase domain in yellow, two amino acids that already belong to the middle domain in blue, the glycine-serine linker in black, the GED in red and the C-term. His tag in pink. Colouring is the same as in figure 8.

The 369 amino acid long GTPase domain-GED fusion protein has a molecular weight of 40 923 Da and a theoretical pI of 6.32 (according to Expasy ProtParam).

Model of the GTPase domain-GED fusion protein

Due to the sequence conservation of 36 % between full length DRP1 and human dynamin (52 % between GTPase domains, Figure 14), a homology model of the DRP1 GTPase domain-GED fusion protein based on the crystal structure of dynamin GG dimer (PDB ID 2X2E) was created (Figure 15).


1 10 20 30 40 50 60
O00429 MEALIPVINKLQDVFNTVGADI-IQLPQIVVVGTQSSGKSSVLESLVGRDLLPRG
Q05193 MGNRGMEDLIPLVNRLQDAFSAIGQNADLDLPQIAVVGGQSAGKSSVLENFVGRDFLPRG
Consensus .....MEaLIPl!NrLQDaFna!Ga#a.i#LPQIaVVGgQSaGKSSVLEnlVGRDlLPRG
61 70 80 90 100 110 120
O00429 TGIVTRRPLILQLVHVSQEDKRKTTGEENGVEAEEWGKFLHTKNKLYTDFDEIRQEIENE
Q05193 SGIVTRRPLVLQLVNATTE----------YAEFLHCKGKKFTDFEEVRLEIEAE
Consensus sGIVTRRPL!LQLVnasqE..............waeFLHcKnKl%TDF#E!RqEIEaE
121 130 140 150 160 170 180
O00429 TERISGNNKGVSPEPIHLKIFSPNVVNLTLVDLPGMTKVPVGDQPKDIELQIRELILRFI
Q05193 TDRVTGTNKGISPVPINLRVYSPHVLNLTLVDLPGMTKVPVGDQPPDIEFQIRDMLMQFV
Consensus T#R!sGnNKG!SPePInLr!%SPnVlNLTLVDLPGMTKVPVGDQPkDIElQIR#irF!
181 190 200 210 220 230 240
O00429 SNPNSIILAVTAANTDMATSEALKISREVDPDGRRTLAVITKLDLMDAGTDAMDVLMGRV
Q05193 TKENCLILAVSPANSDLANSDALKVAKEVDPQGQRTIGVITKLDLMDEGTDARDVLENKL
Consensus sneNciILAVsaANsD$AnS#ALK!arEVDP#GrRTiaVITKLDLMDaGTDArDVLenrl
241 250 260 270 280 290 300
O00429 IPVKLGIIGVVNRSQLDINNKKSVTDSIRDEYAFL--QKKYPSLANRNGTKYLARTLNRL
Q05193 LPLRRGYIGVVNRSQKDIDGKKDITAALAAERKFFLSHPSYRHLADRMGTPYLQKVLNQQ
Consensus iPlrrGiIGVVNRSQlDI#nKKd!TaaiaaEraFl..qkkYrhLA#RnGTkYLartLNrq
301 310 320 330 340 350 360
O00429 LMHHIRDCLPELKTRINVLAAQYQSLLNSYGEPVDD----KSATLLQLITKFATEYCNTI
Q05193 LTNHIRDTLPGLRNKLQSQLLSIEKEVEEYKNFRPDDPARKTKALLQMVQQFAVDFEKRI
Consensus LmnHIRDcLPeLrnri#sqaaqi#kel#eYg#frdD....KsaaLLQ$!qqFAt#%cnrI
361 370 380 390 400 410 420
O00429 EGTAKYIETSELCGGARICYIFHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALFV
Q05193 EGSGDQIDTYELSGGARINRIFHERFPFELVKMEFDEKELRREISYAIKNIHGIRTGLFT
Consensus EGsadqI#TsELcGGARInrIFHErFgreLekm#fdegenrr#IltAIrNahGiRpaLFt
421 430 440 450 460 470 480
O00429 PEVSFELLVKRQIKRLEEPSLRCVELVHEEMQRIIQHCSNYSTQELLRFPKLHDAIVEVV
Q05193 PDMAFETIVKKQVKKIREPCLKCVDMVISELISTVRQC----TKKLQQYPRLREEMERIV
Consensus P#maFEliVKrQ!KrirEPcLrCV#$VheE$qri!rqC....TqeLqr%PrLr#aier!V
481 490 500 510 520 530 540
O00429 TCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFADACGLMNNNIEEQRRNRLARELPSAV
Q05193 TTHIREREGRTKEQVMLLIDIELAYMNTNHEDFIGFANAQQRSNQMNKKKTSGNQDEILV
Consensus TchiReRegrTnEqVhnL!aIELAYiNTnHeDFadaanaq#rnn#e#rrnrlar#deiaV
541 550 560 570 580 590 600
O00429 SRDKSSKVPSA--LAPASQEPSPAASAEADGKLIQDSRRETKNVASGGGGVGDGVQEPTT
Q05193 IRKGWLTINNIGIMKGGSKEYWFVLTAENLSWYKDDEEKEKKYMLSVDNLKLRDVEKGFM
Consensus iRdgslk!nna..$agaSqEpsfaasAEadgkli#DerrEkKnmaSgdngkgrdV#egfm
601 610 620 630 640 650 660
O00429 GN----------WRGMLKTSKAEELLAEEKSKPIPIMPASPQKGHAVNLLDVPVPVAR--
Q05193 SSKHIFALFNTEQRNVYKDYRQLELACETQEEVDSWKASFLRAGVYPERVGDKEKASETE
Consensus gn.........qRnmlKdsraeELaaEeqeepdpikaaflraGhap#rlddkekaaer..
661 670 680 690 700 710 720
O00429 ---------KLSAREQRDCEVIERLIKSYFLIVRKNIQDSVPKAVMHFLVNHVKDTLQSE
Q05193 ENGSDSFMHSMDPQLERQVETIRNLVDSYMAIVNKTVRDLMPKTIMHLMINNTKEFIFSE
Consensus ....k$dare#R#cEtIrrL!dSYmaIVrKn!rDlmPKa!MHl$!NntK#fiqSE
721 730 740 750 760 770 780
O00429 LVGQLYKSSLLDDLLTESEDMAQRRKEAADMLKALQGASQIIAEIRETHLW
Q05193 LLANLYSCGDQNTLMEESAEQAQRRDEMLRMYHALKEALSIIGDINTTTVSTPMPPPVDD
Consensus Lla#LYkcgdq#dL$eESa#qAQRRdEaarMlhALqeAlqIIa#IreThls.........
781 790 800 810 820 830 840
O00429
Q05193 SWLQVQSVPAGRRSPTSSPTPQRRAPAVPPARPGSRGPAPGPPPAGSALGGAPPVPSRPG
Consensus ....
841 850 860 870 880 884
O00429
Q05193 ASPDPFGPPPQVPSRPNRAPPGVPSRSGQASPSRPESPRPPFDL
Consensus .....

Figure 14. Sequence alignment of human dynamin 1 (UniProt number: Q05193) with human dynamin-related protein 1 (UniProt number: O00429) created with Multiple sequence alignment with hierarchical clustering (F. Corpet, 1988, Nucl. Acids Res., 16 (22), 10881-10890). Identical amino acid residues are shown in red.

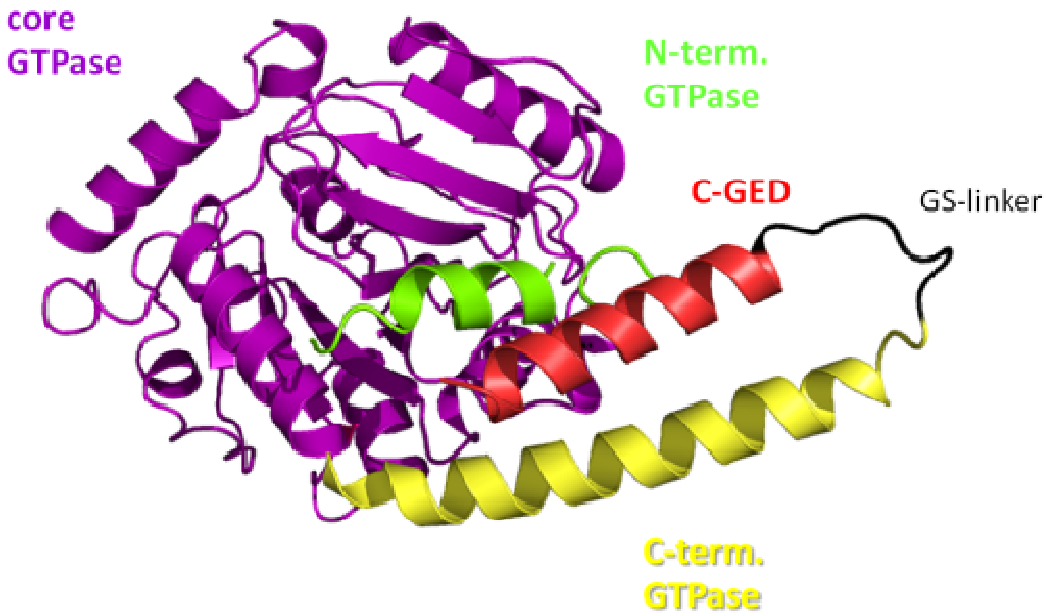


Figure 15. Homology model of the GTPase domain-GED fusion protein. Model is based on PDB entry 2X2E (Chappie et al, 2010) and was created using FFAS. The N-terminal part of the GTPase domain is coloured in green, the core in purple and the C-terminal part in yellow. GS linker is shown in black and the C-terminal part of the GED in red. GS linker – Glycine-serine linker, GED – GTPase effector domain. Figure was created with Pymol (Version 1.3).

3.3. CLONING

Cloning of DNM1 and DRP1 constructs worked very well and all constructs were checked by sequencing.

3.4. PROTEIN EXPRESSION

3.4.1. EXPRESSION OF DNM1 CONSTRUCTS E 59 – E 62

Whereas expression of DNM1 wildtype proteins (E 59 and E 60) and the G385D mutation protein E 62 failed, mutation protein E 61 showed moderate expression.

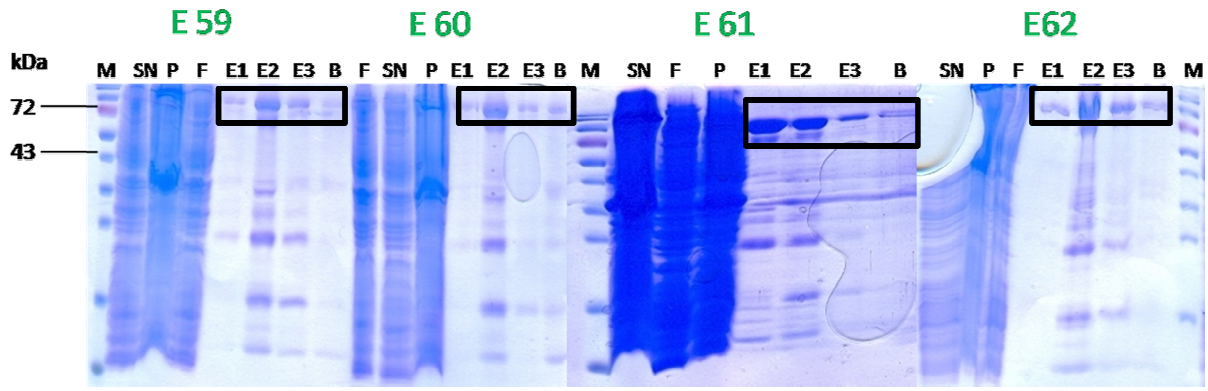


Figure 16. SDS-PAGE of DNM1 wildtype and mutation proteins after IMAC. M – marker, SN – supernatant, P – pellet, F – flow-through, E – elution, B – beads. E 59 and E 61: 86 kDa, E 60 and E 62: 88 kDa.

3.4.2. EXPRESSION OF DRP1 CONSTRUCTS E 63 – E 72

Expression of short GTPase domain-GED fusion proteins (E 63 and E 64) worked very well, whereas the expression of longer fusion proteins (E 65 and E 66) did not show high protein amounts.

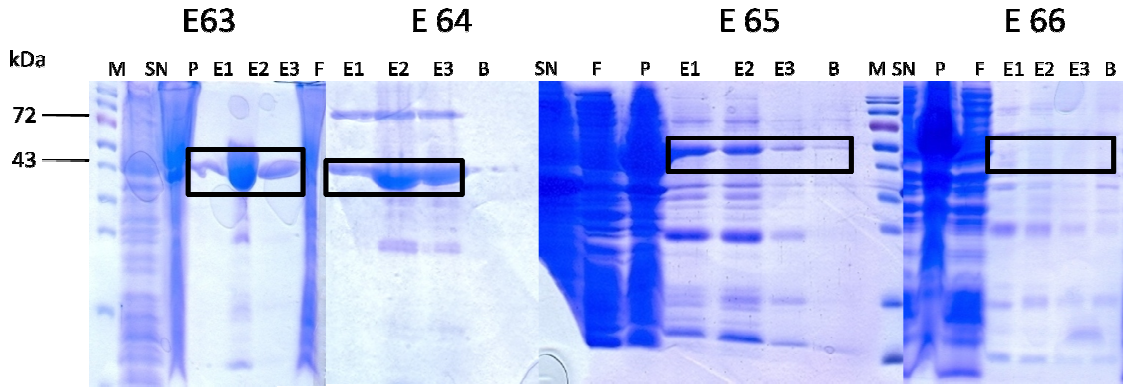


Figure 17. SDS-PAGE of DRP1 GTPase domain-GED fusion proteins after IMAC. M – marker, SN – supernatant, P – pellet, F – flow-through, E – elution, B – beads. E 63: 41 kDa, E 64: 43 kDa, E 65:49 kDa, E 66: 51 kDa.

The expression of mutation constructs (E 67 – E 69) yielded a very high protein amount.

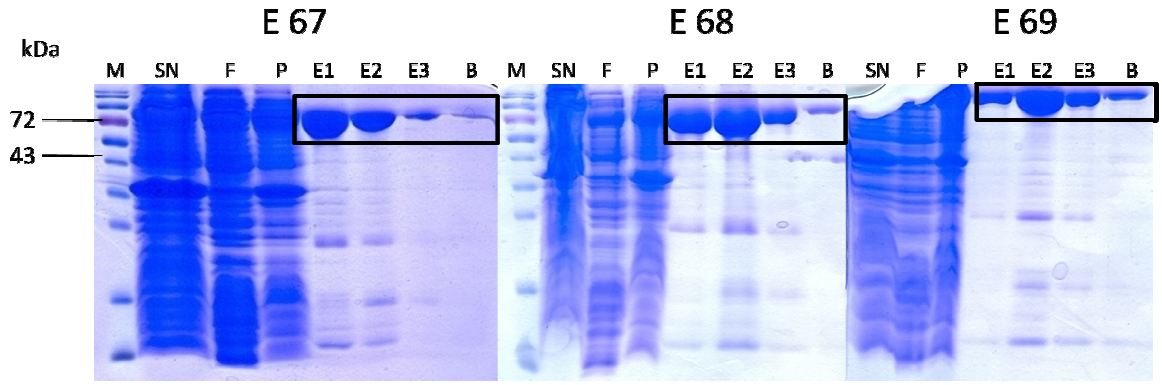


Figure 18. SDS-PAGE of DRP1 mutation constructs E 67, E 68 and E 69 after IMAC. M – marker, SN – supernatant, P – pellet, F – flow-through, E – elution, B – beads. E 67, E 68 and E 69: 83 kDa.

Likewise, expression of GTPase domain constructs (E 70 – E 72) was very successful.

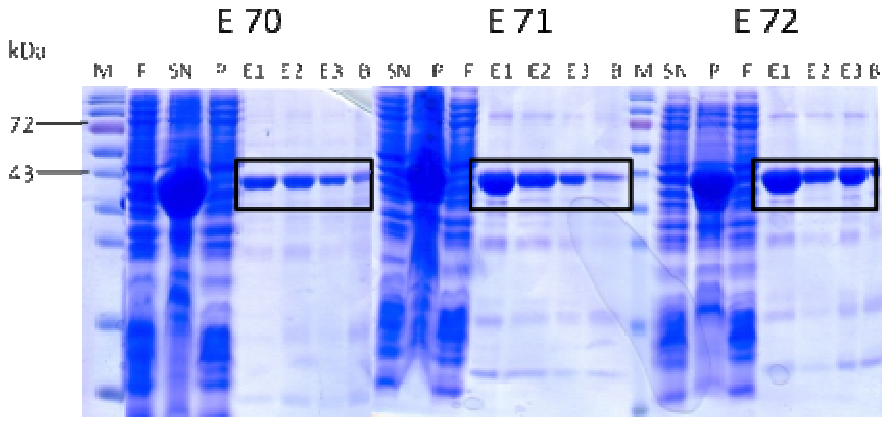


Figure 19. SDS-PAGE of DRP1 GTPase domain constructs E 70, E 71 and E 72 after IMAC. M – marker, SN – supernatant, P – pellet, F – flow-through, E – elution, B – beads. E 70, E 71 and E 72: ~39 kDa.

3. 5. EXPRESSION AND SUBSEQUENT PURIFICATION OF THE GTPASE DOMAIN-GED FUSION PROTEIN

As mentioned above, the GTPase domain-GED fusion protein consists of a N-terminal GTPase domain that was fused to the C-terminal part of the GED via a glycine-serine linker.

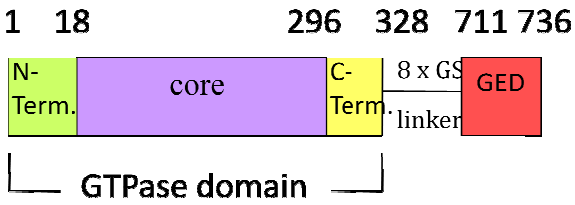


Figure 20. Domain architecture of the GTPase domain-GED fusion protein.
The C-terminal His-tag is not shown. GS linker – Glycine-serine linker, GED – GTPase effector domain.

The protein expression worked very well (Figure 21).

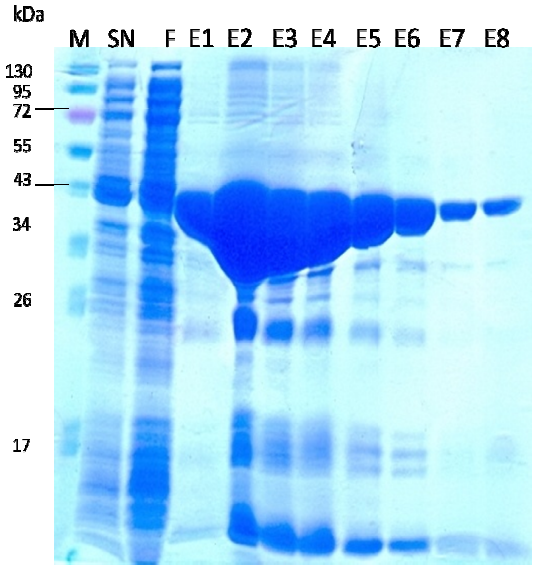


Figure 21. SDS-PAGE of the GTPase domain-GED fusion protein. M – marker, SN – supernatant, F – flow-through, E – elution.

Since the expression level of this protein showed the highest yield, a second purification step was carried out:

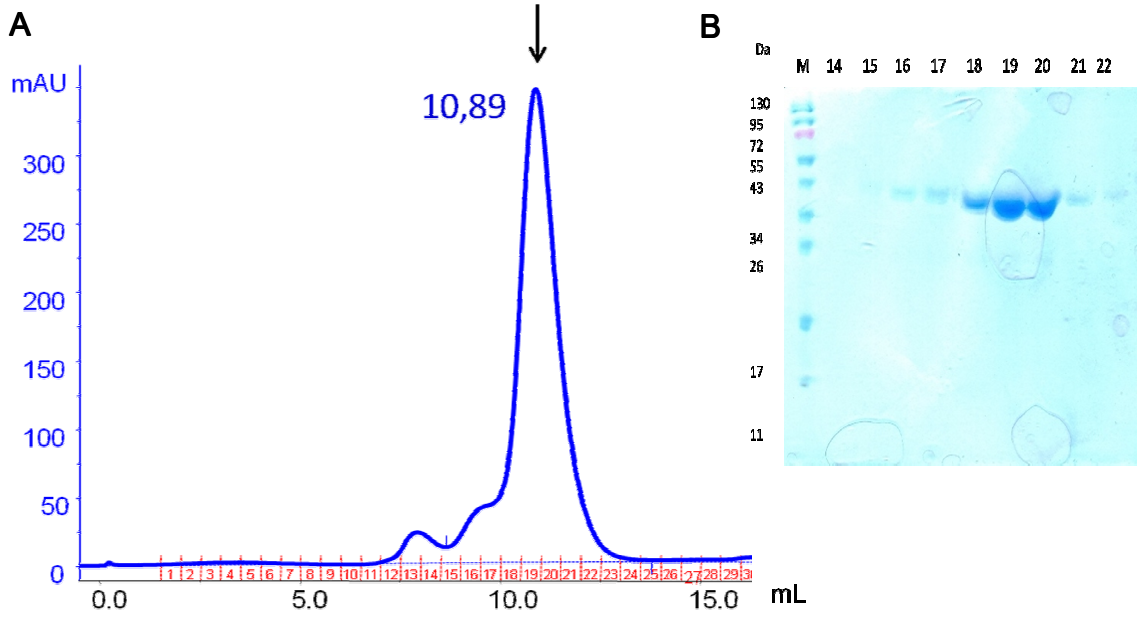


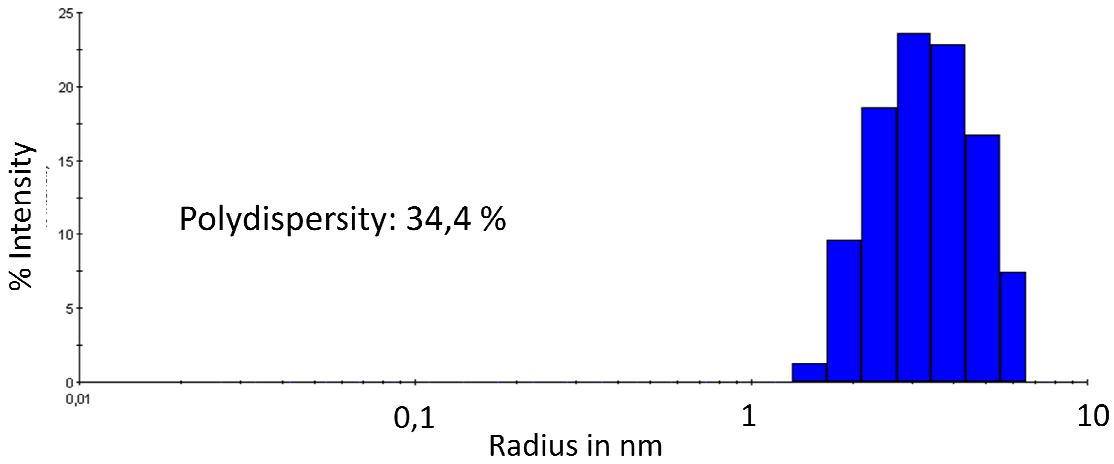
Figure 22. A. SEC analysis of the GTPase domain-GED fusion protein. The protein elutes at a retention volume of 10,89 mL. **B.** SDS PAGE of the GTPase domain-GED fusion protein after size exclusion chromatography.

Most of the protein eluted at a retention volume of 10,89 mL which corresponds to a monomer when compared with a previously performed standard curve (see Material and Methods). SDS-PAGE analysis shows that the protein is 99% pure. Purity is an important determinant for successful crystallization.

3.6. DLS ANALYSIS

In order to evaluate the homogeneity of the protein sample and to decide whether or not the protein sample should be sterile filtered prior to crystallization, dynamic light scattering was performed [34, 35, 36].

A.



B.

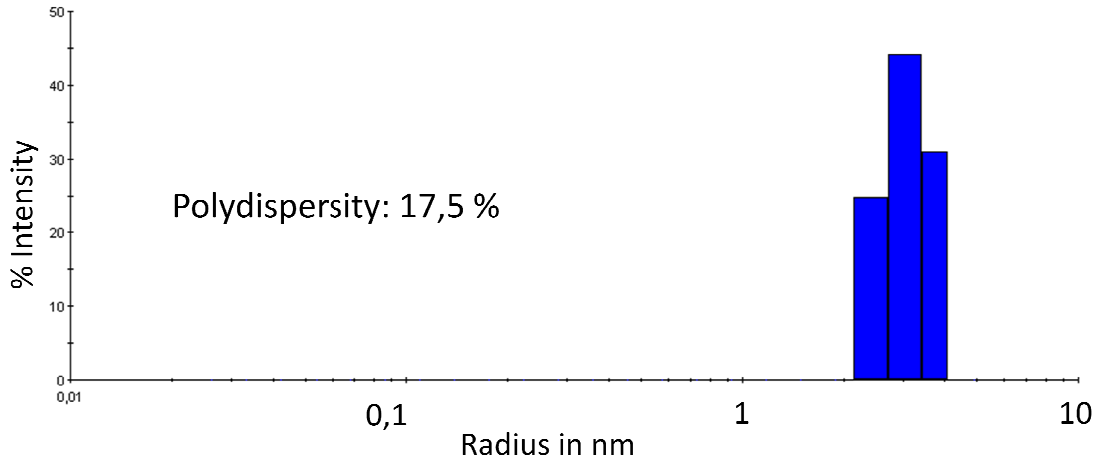


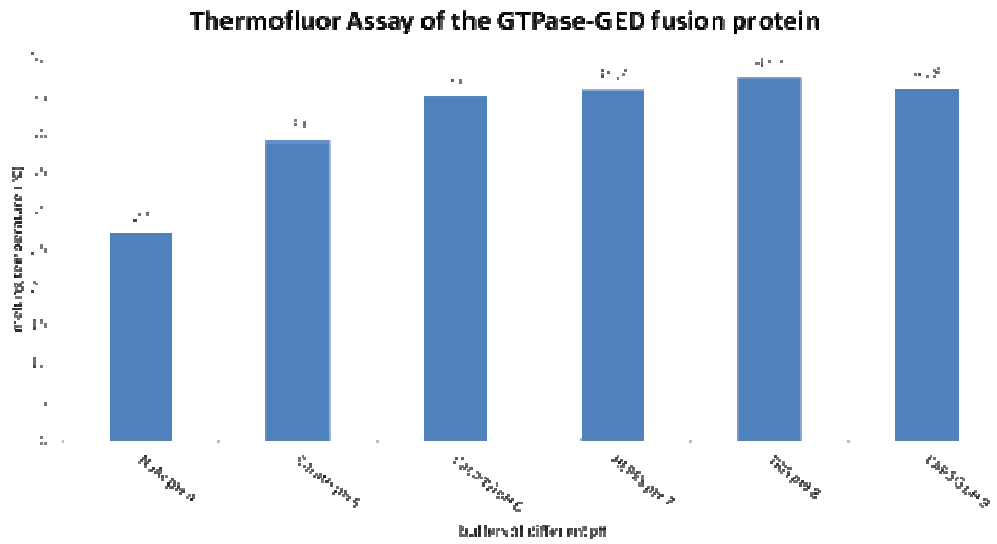
Figure 23. DLS analysis of the GTPase-GED fusion protein sterile filtered prior to measurement (**A.**) or left untreated (**B.**).

The size distribution histograms of both samples show a single peak (monomodal size distribution). The polydispersity of the untreated protein is lower (17,5%) than that of the sterile filtered sample (34,4%). Thus, the unfiltered protein was used for crystallization experiments.

3.7. THERMOFLUOR ASSAY

In order to analyze the stability of the protein after size exclusion chromatography in different buffers, a thermofluor assay was performed using 3,7 µM DRP1 fusion protein:

A.



B.

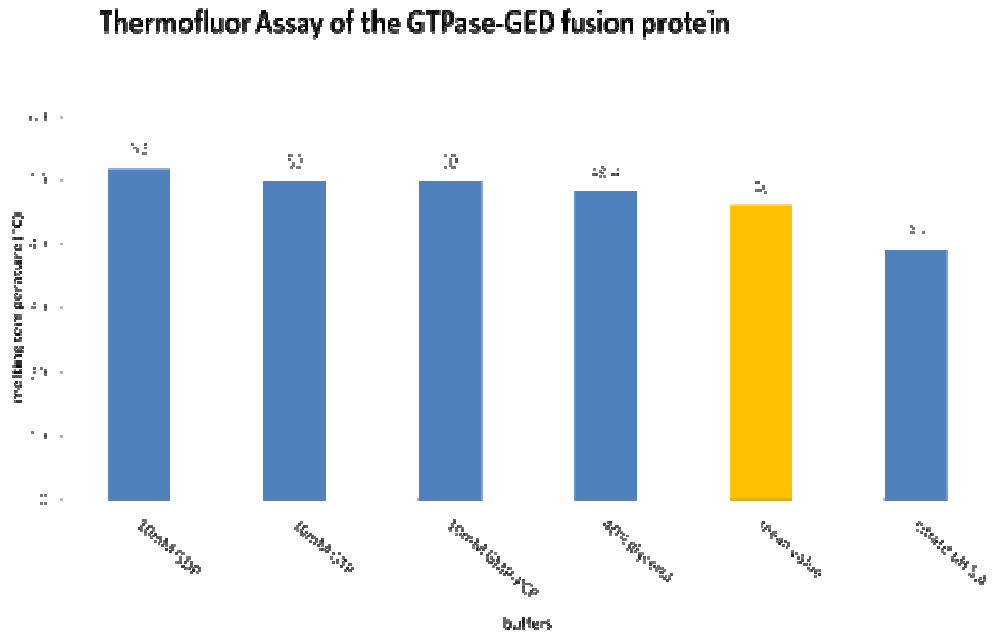


Figure 24. Melting temperature diagram of the DRP1 GTPase domain-GED fusion protein in SEC buffer after size exclusion chromatography in buffers of different pH (**A.**) and with additives (**B.**).

The GTPase domain-GED fusion protein is more stable in buffers around neutral pH. Acidic pH has a negative effect on the stability of the protein. Moreover, it is more stable when guanine nucleotide analogs GDP, GTP or GMP-PCP - a non hydrolyzable GTP analog - are added. Addition of 40 % Glycerol shows a similar effect.

3.8. OLIGOMERIZATION STATES OF THE DRP1 GTPASE DOMAIN-GED FUSION PROTEIN UPON ADDITION OF GUANINE NUCLEOTIDE ANALOGS

Before incubation of the DRP1 GTPase domain-GED fusion protein with guanine nucleotide analogs, a buffer exchange into SEC 2 buffer (see Appendix) was performed. The protein was incubated with either 1 mM GDP, 1 mM GTP or 1 mM GMP-PCP for 30 minutes at room temperature and then analyzed by SEC.
In parallel, a buffer exchange into a buffer containing 4 mM $MgCl_2$, 2 mM EGTA and 1 mM DTT (SEC 3 buffer, see Appendix) was performed. The DRP1 GTPase domain-GED fusion protein was incubated with transition state mimic 2 mM GDP, 2 mM $AlCl_3$ and 20 mM NaF for 30 minutes at 37°C and then analyzed by SEC. The experimental setup was designed according to Chappie et al in 2010 [16].

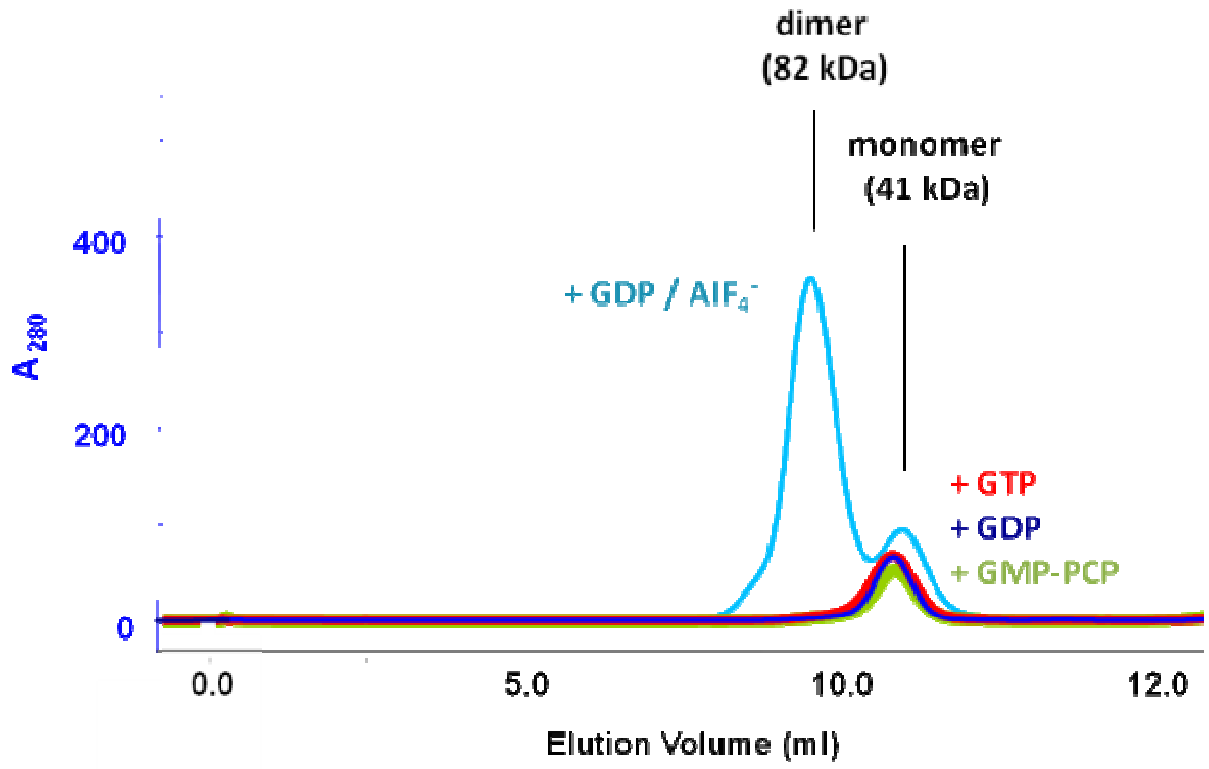


Figure 25. SEC analysis of the GTPase domain-GED fusion protein in the presence of either 1 mM GDP (blue), 1 mM GTP (red), 1 mM GMP-PCP (green) or transition state mimic (2mM GDP, 2mM $AlCl_3$ and 20mM NaF, light blue).

As shown in figure 25, the addition of neither GDP nor GTP nor GMP-PCP alters the oligomerization state of the GTPase domain-GED fusion protein: The monomer eluted again at a retention volume of ~10,9 mL. However, the DRP1 transition state mimic eluted mainly as a dimer at a retention volume of ~9,4 mL.

3.9. GTPASE ASSAY

For further protein characterization, a buffer exchange into 25 mM HEPES, 25 mM PIPES and 150 mM NaCl (GTPase assay buffer, see Appendix) was performed after size exclusion chromatography. Subsequently, DRP1 full length protein and GTPase domain-GED fusion protein were assayed for their GTPase activity with a continuous, regenerative coupled GTPase assay for dynamin-related proteins [31]:

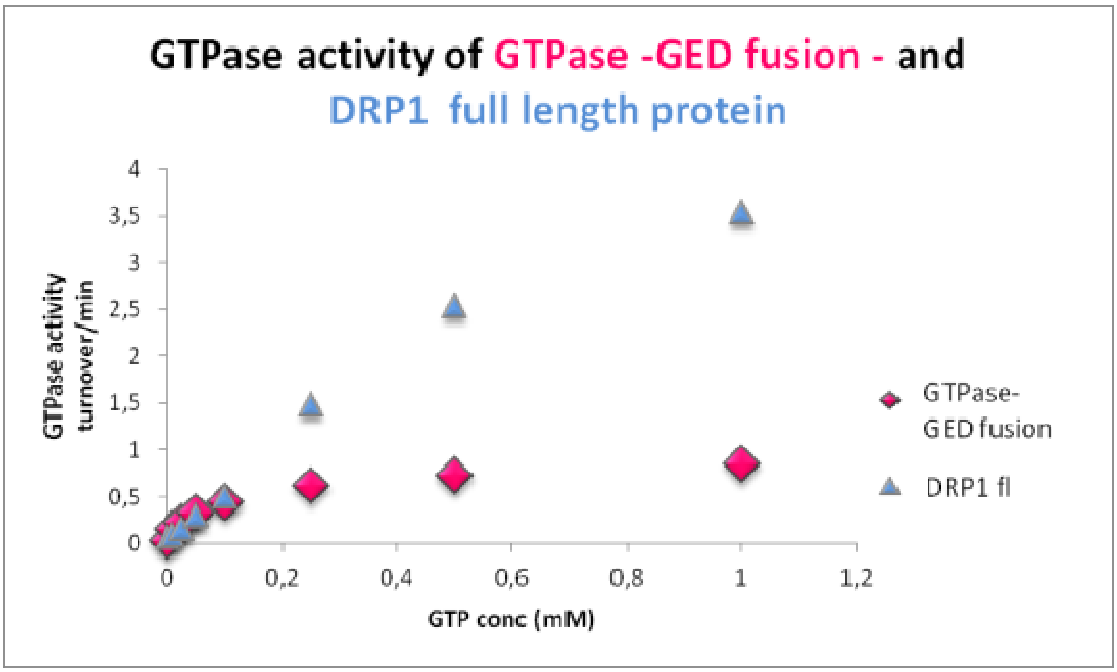


Figure 26. GTPase assay of wildtype DRP1 and GTPase domain-GED fusion protein.

As shown in figure 26, the wildtype protein exhibits a fivefold higher GTPase activity than the GTPase domain-GED fusion protein at a GTP concentration of 0,5 mM.

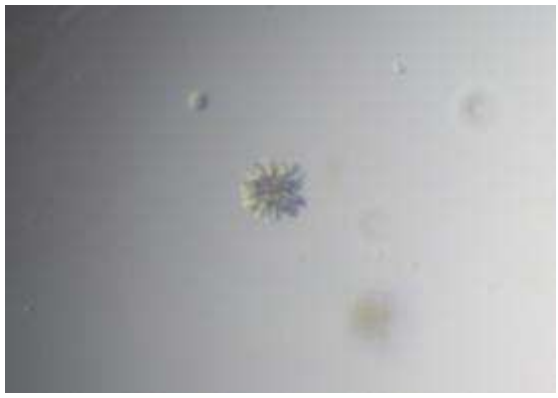
3.10. CRYSTALLIZATION

The GTPase-GED fusion protein used for crystallization was monomeric and in SEC buffer. Initial crystallization screening was performed using several commercially available crystallization screens.

Initial hits were obtained at following conditions:

- 1. 0,1 M sodium citrate pH 5,5 20 % (w/v) PEG 3000 (day 1)
- 2. 0,2 M lithium sulfate 0,1 M Bis-Tris pH 5,5 25% (w/v) PEG 3350 (day 11)

A.



B.

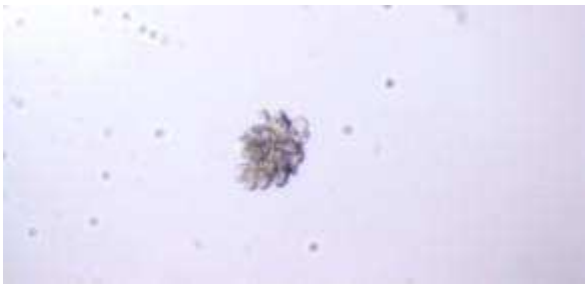


Figure 27. GTPase domain-Ged fusion protein crystals. **A.** Initial hit observed after 1 day in 0,1 M sodium citrate pH 5,5 20 % (w/v) PEG 3000. **B.** Initial hit in 0,2 M lithium sulfate, 0,1 M Bis-Tris pH 5.5, 25 % (w/v) PEG 3350 after 11 days. 10,4 mg/mL monomeric protein was used.

First, the condition containing lithium sulfate and Bis-Tris was used. Initial hits were refined manually, performing hanging - and sitting-drop vapor diffusion techniques [37, 38, 39]. For fine screening protein and precipitant concentration, and pH were modified [40, 41, 42].

Figure 28. Refined GTPase domain-GED fusion protein crystals, grown in 0,2 M lithium sulfate, 0,1 M Bis-Tris pH 5 and 25% (w/v) PEG 3350. 10,4 mg/mL monomeric protein was used.

Thin plates from these crystals were broken and flash-frozen in liquid nitrogen. These crystals showed diffraction (see X-ray diffraction analysis), however, the quality of the data was not good enough to enable further data processing. Thus, further optimization was attempted. Unfortunately, reproduction of these crystals failed. Therefore, refinement of crystals in the condition containing sodium citrate was performed (Figure 29).

A. **B.**

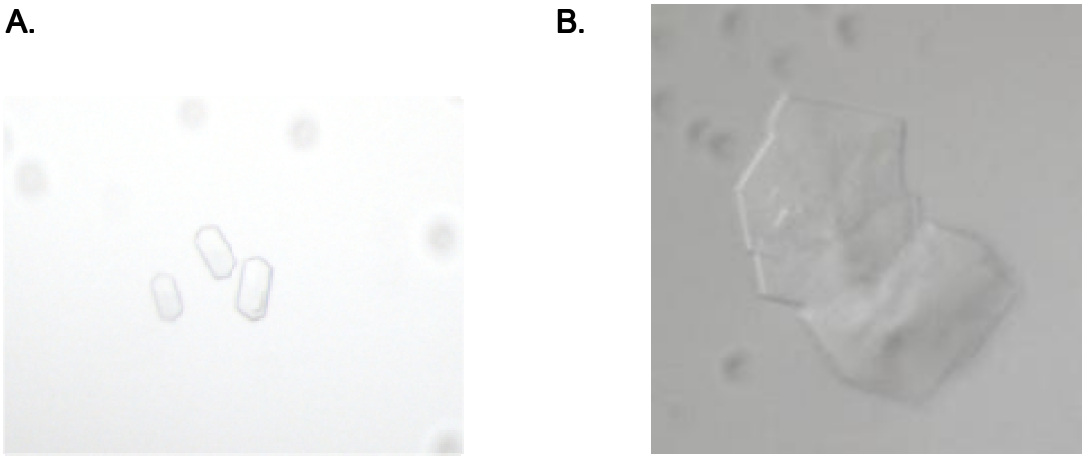


Figure 29. A. and **B.** show refined crystals of 9,6 mg/mL monomeric GTPase domain-GED fusion protein.

3.11. X-RAY DIFFRACTION ANALYSIS

Initial diffraction experiments were performed at an in-house rotating anode facility, to check that the crystals are protein and not salt.

An X-ray diffraction data set was collected on beamline ID14-4 at the ESRF Grenoble to a resolution of 4 Å. We thank Ulrich Eckhard for data collection.The orthorhombic crystals were indexed with space group P2 and unit cell parameters a = 42.15, b = 53.31, c = 150.54 Å. Since the collected data showed only limited diffraction due to ice rings and high anisotropy, further data processing failed.

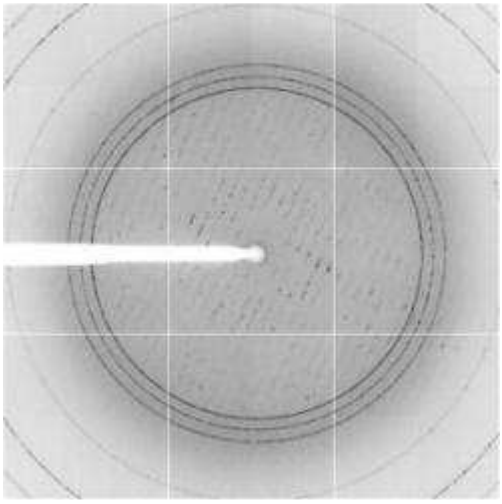


Figure 30. 1.0° Oscillation image of the DRP1 GTPase domain-GED fusion protein crystal collected at ESFR Grenoble.Thanks for data collection to Ulrich Eckhard.

DRP1 GTPase domain-GED fusion protein	
Space Group	P2
Cell dimensions	
a (Å)	42,15
b (Å)	53,31
c (Å)	150,54
α, β, γ (°)	90/90/90
Resolution (Å)	4

Table 11. Data statistics of the DRP1 GTPase domain-GED fusion protein.

4. DISCUSSION

In order to characterize dynamin related protein 1, several constructs were designed: Out of 10 constructs, 9 could be successfully expressed in E. coli and purified by IMAC. 4 constructs were further purified using SEC. For all DNM1 constructs, expression in E. coli did not yield sufficient protein amounts.
For several reasons just the DRP1 GTPase domain-GED fusion protein (construct E 63) was characterized: Expression of the GTPase domain-GED fusion protein showed a high protein yield (approximately 3,3 mg/L culture). Unlike full length constructs, this protein contains no flexible linker region which might prevent crystallization. The GTPase domain-GED fusion protein is more stable than the GTPase domain constructs, because backfolding of the C-terminus of the GED is known to stabilize the protein. Moreover, the crystal structure of the corresponding construct in dynamin 1 was successfully solved by Chappie et al in 2010 [16].

It could be established that the GTPase domain-GED fusion protein is a functional GTPase which behaves as expected: The GTPase activity is lower than that of the wildtype protein due to impaired oligomerization and SEC analysis revealed dimer formation upon incubation with transition state mimics GDP, NaF and $AlCl_3$. Thus, the GTPase domain-GED fusion protein was further analyzed by DLS, Thermofluor, GTPase assay and crystallization. Furthermore, analytical SEC runs were performed upon addition of guanine nucleotide analogs and transition state mimics:

Dynamic light scattering analysis of the GTPase domain-GED fusion protein yielded a polydispersity of 17,5%, a value that can be considered relatively low. As a general rule, it can be stated that a lower polydispersity displays a higher homogeneity of the sample and therefore a higher probability of crystallization. For DRP1, sterile filtration prior to crystallization was less favorable due to a higher degree of inhomogeneity. However, it can be assumed that this may be caused by interactions of the protein with the hydrophobic membrane of the filter, which may lead to partial unfolding.

In conformity with the size exclusion chromatography results, DLS showed that the majority of the protein exists as one distinct oligomerization state, namely as monomer.

Thermofluor assays showed that the GTPase domain-GED fusion protein is most stable in buffers with pH around 8 and upon incubation with 10 mM GDP,10 mM GTP 10 mM GMP-PCP and 40 % Glycerol, respectively.

Analytical SEC runs revealed that the oligomerization state of the GTPase domain-GED fusion protein is not altered upon addition of either GDP, GTP or GMP-PCP. In the presence of GDP, NaF and $AlCl_3$, however, DRP1 fusion protein dimerized. This fact implies that the DRP1 GTPase-GED fusion construct is functional and behaves like previously published for the human dynamin [16].

Chappie et al [16] confirmed that only upon assembly of two semi-functional GTPase domains, the contribution of amino acid residues from both results in a fully functional catalytic center explaining a key feature of dynamin superfamily members, namely the assembly-stimulated GTPase activity.

Analysis of the GTPase activity showed that the GTPase domain-GED fusion protein exhibits a fivefold lower activity than the wildtype. Since the fusion protein lacks the middle domain, which plays an important role in self-assembly, it can only form dimers, but not higher oligomers, impairing assembly-stimulated GTPase activity.

In summary, the successful expression and purification of the monodisperse DRP1 GTPase domain-GED fusion protein that existed as a stable monomer was achieved. The protein shows GTPase activity and is able to dimerize in presence of the transition state mimics. Furthermore, the successful crystallization of the GTPase domain-GED fusion protein was achieved; the most important step for solving its crystal structure. Additional experiments are underway.

5. SUMMARY

The aim of this study was the biochemical and biophysical characterization of human dynamin-related protein 1 (DRP1), the main mitochondrial division factor.

Mitochondria are considered to play a crucial role in energetic metabolism, apoptosis and calcium homeostasis. In oder to accomplish their functions, they undergo frequent fission and fusion, which is very important in tissues with high dependence on energy supply, such as neurons. Mitochondrial dynamics are mediated by a group of large, self-assembling GTPases; the most important in humans are DRP1 and fusion factors mitofusins and OPA1.
Obviously, dysfunction or misregulation of mitochondrial dynamics leads to neurodegenerative disorders like Charcot-Marie-Tooth disease, Alzheimer´s -, Parkinson´s - and Huntington´s disease.
In the last-mentioned disorder, elevated levels of GTPase activity of DRP1 have been shown to result in excessive mitochondrial fragmentation which causes neuronal cell death.
Thus, DRP1 might be a new target for drug design in treatment of this disease. Therefore solving its crystal structure is of high clinical relevance and great scientific interest.

Here we report the successful cloning, expression and crystallization of the DRP1 GTPase domain-GED fusion protein. Initial diffraction data are promising and we are confident that its crystal structure will be soon solved.

6. ZUSAMMENFASSUNG

Das Ziel dieser Studie war die Charakterisierung des Proteins DRP1 (dynamin related protein 1), der Hauptfaktor bei der mitochondrialen Teilung, auf biochemischer und biophysikalischer Ebene.
Mitochondrien spielen unter anderem im Energiehaushalt, in der Apoptose und der Homöostase von Kalzium eine entscheidende Rolle. Um diese Funktionen zu erfüllen, teilen sie sich kontinuierlich und verschmelzen wieder. Dies ist besonders wichtig in Gewebe, das stark von der Energieversorgung durch diese Organellen abhängt, wie die Neuronen. Dieser dynamische Prozess wird von einer Gruppe von großen GTPasen ausgeführt, welche selbst zu höheren Oligomeren assemblieren. Zu den wichtigsten Vertretern im Menschen zählen DRP1 und die bei Verschmelzungsprozessen involvierten Proteine, OPA1 und Mitofusine.
Naturgemäß führt eine Funktionsstörung oder ein Fehler in der Regulation der mitochondrialen Dynamik zu degenerativen Krankheiten des Nervensystems wie zum Beispiel: Charcot-Marie-Tooth-Syndrom, Alzheimer-Krankheit, Parkinson-Krankheit und Chorea Huntington.
In der letztgenannten Störung führt eine erhöhte GTPase Aktivität von DRP1 zu einer übermäßigen Fragmentierung von Mitochondrien, welche in weiterer Folge das Absterben von Nervenzellen verursacht.

DRP1 spielt daher eine bedeutende Rolle bei der Suche nach Medikamenten gegen Chorea Huntington. Und aus eben diesem Grund ist die Aufklärung seiner Struktur nicht nur von großem, wissenschaftlichen Interesse sondern auch von bedeutender, klinischer Relevanz.

Diese Arbeit berichtet von der erfolgreichen Klonierung, Expression, Reinigung und Kristallisation des DRP1-Proteins, in welchem die GTPase Domäne mit der GED fusioniert wurde. Erste Untersuchungsergebnisse mittels Röntgenstrukturanalyse sind sehr vielversprechend und wir sind zuversichtlich, dass die Kristallstruktur von DRP1 in naher Zukunft gelöst wird.

CURRICULUM VITAE

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PRACTICAL EXPERIENCE

07/2011 – dato

University of **Salzburg**, Department of Molecular Biology, *Protein Engineering Biotechnology:* Protein expression and purification, crystallization, x-ray crystallography

Master thesis: "*Functional characterization of human DRP1*" („*Funktionelle Charakterisierung von humanen DRP1*")

01/2008 - 03/2008

Max F. Perutz Laboratories (MFPL), Vienna Biocenter, *Signaling and gene expression in inflammation:* Cell culture, bacterial culture (Streptococcus pyogenes), fluorescence microscopy, bone marrow preparation

02/2007 – 08/2007

AKH Wien, **Medical University of Vienna**, research assistant, *Tissue Engineering*: Cell culture, western blot, elisa, establishment of qPCR, experience in clean room

STUDIES

03/2011 - dato	Master studies of *Immunobiology and Microbiology*, Faculty of Molecular Biology, University of Vienna
03/2011	Shift to the Bachelor and Master Degree course in *Microbiology and Immunobiology*, Faculty of Molecular Biology, University of Vienna, Conferment of **Bachelor of Science (B.Sc.)**
SS 2005 – WS 2008	Diploma studies *Genetics and Microbiology*, major field of studies: *Immunology and Virology* minor field of studies: *Molecular Microbiology*
31.05.2005	1. Diplomprüfungszeugnis (finish of the first part of studies)
2003 – 2008	Diploma studies of Biology, **University of Vienna**

EDUCATION

11.06.2003	**Matura** mit ausgezeichnetem Erfolg (school leaving examination, graduation with honors)
1995 – 2003	Bundesrealgymnasium Körnerschule (academic secondary school) Linz
1991 – 1995	Volksschule Linz

SKILLS

Languages:	German	native
	English	fluent (written and spoken)
	French, Spanish	basics
EDV:	Microsoft Windows, - Office	excellent
	Lotus Notes und NCBI-PubMed	excellent
	Protein databases (UniProtKB, Prosite, RCSB Protein Data Bank)	good

Linz, April, 1 st, 2012

APPENDIX

1. SEQUENCES

1.1. DNA SEQUENCE OF DRP1

DNA sequence was obtained from NCBI (NCBI Reference Sequence: NM_012062.3)

```
ORIGIN
        1 gcatggcctg ccgggagggg gcaggtagcc ggcgggcccg gtccaatggg tgccggcttc
       61 cgaggagagg gcggaggaga ggaggaagga ggcgaactgt gggccccggc cccattcatt
      121 gccgtggccg gcgggcactg gggccccgtg ttttcagagt catggaggcg ctaattcctg
      181 tcataaacaa gctccaggac gtcttcaaca cggtgggcgc cgacatcatc cagctgcctc
      241 aaatcgtcgt agtgggaacg cagagcagcg gaaagagctc agtgctagaa agcctggtgg
      301 ggagggacct gcttcccaga ggtactggaa ttgtcacccg gagacctctc attctgcaac
      361 tggtccatgt ttcacaagaa gataaacgga aaacaacagg agaagaaaat ggggtggaag
      421 cagaagaatg gggtaaattt cttcacacca aaaataagct ttacacggat tttgatgaaa
      481 ttcgacaaga aattgaaaat gaaacagaaa gaatttcagg aaataataag ggagtaagcc
      541 ctgaaccaat tcatcttaag atttttttcac ccaacgttgt caatttgaca cttgtggatt
      601 tgccaggaat gaccaaggtg cctgtaggtg atcaacctaa ggatattgag cttcaaatca
      661 gagagctcat tcttcggttc atcagtaatc ctaattccat tatcctcgct gtcactgctg
      721 ctaatacaga tatggcaaca tcagaggcac ttaaaatttc aagagaggta gatccagatg
      781 gtcgcagaac cctagctgta atcactaaac ttgatctcat ggatgcgggt actgatgcca
      841 tggatgtatt gatgggaagg gttattccag tcaaacttgg aataattgga gtagttaaca
      901 ggagccagct agatattaac aacaagaaga gtgtaactga ttcaatccgt gatgagtatg
      961 cttttcttca aaagaaatat ccatctctgg ccaatagaaa tggaacaaag tatcttgcta
     1021 ggactctaaa caggttactg atgcatcaca tcagagattg tttaccagag ttgaaaacaa
     1081 gaataaatgt tctagctgct cagtatcagt ctcttctaaa tagctacggt gaacccgtgg
     1141 atgataaaag tgctacttta ctccaactta ttaccaaatt tgccacagaa tattgtaaca
     1201 ctattgaagg aactgcaaaa tatattgaaa cttcggagct atgcggtggt gctagaattt
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     1321 ttaacactat tgacattttg actgccatta gaaatgctac tggtcctcgt cctgctttat
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     1441 ccagcctccg ctgtgtggaa ctggttcatg aggaaatgca aaggatcatt cagcactgta
     1501 gcaattacag tacacaggaa ttgttacgat ttcctaaact tcatgatgcc atagttgaag
     1561 tggtgacttg tcttcttcgt aaaaggttgc ctgttacaaa tgaaatggtc cataacttag
     1621 tggcaattga actggcttat atcaacacaa aacatccaga ctttgctgat gcttgtgggc
     1681 taatgaacaa taatatagag gaacaaagga gaaacaggct agccagagaa ttaccttcag
     1741 ctgtatcacg agacaagtct tctaaagttc caagtgcttt ggcacctgcc tcccaggagc
     1801 cctccccccgc tgcttctgct gaggctgatg gcaagttaat tcaggacagc agaagagaaa
     1861 ctaaaaatgt tgcatctgga ggtggtgggg ttggagatgg tgttcaagaa ccaaccacag
     1921 gcaactggag aggaatgctg aaaacttcaa aagctgaaga gttattagca gaagaaaaat
     1981 caaaacccat tccaattatg ccagccagtc cacaaaaagg tcatgccgtg aacctgctag
     2041 atgtgccagt tcctgttgca cgaaaactat ctgctcggga acagcgagat tgtgaggtta
     2101 ttgaacgact cattaaatca tattttctca ttgtcagaaa gaatattcaa gacagtgtgc
     2161 caaaggcagt aatgcatttt ttggttaatc atgtgaaaga cactcttcag agtgagctag
     2221 taggccagct gtataaatca tccttattgg atgatcttct gacagaatct gaggacatgg
     2281 cacagcgcag gaaagaagca gctgatatgc taaaggcatt acaaggagcc agtcaaatta
     2341 ttgctgaaat ccgggagact catctttggt gaagagaact atgtaatact gagactttgt
     2401 tgactcaaaa cttgctagtt actgcctacc tgagtagaat cttatttatg aactcctgtg
     2461 tattgcaatg gtatgaatct gctcatgtgg agactggcta taaactgaaa agtgtattcc
     2521 aaattgcaga acacatcaca catttaatcc aaataataaa tggctgtttc taaagtttcc
```

```
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2701 aataatcttt gttagtagtc ttaaagctgc tgccatagtc ctccaagaag aaagcaccaa
2761 gacaacattt catatgacta taatgcatgt actatataag ctgatctggc tttgaaagat
2821 gtgagttggc aagttcctca catagagtca ttgtattcca cctgtccttc aatttagttt
2881 tttctgagct tctttgcagc ctttgatgtg tttttaagaa agctgaatgc acaagaggat
2941 ctgtgacact gacatggctg tggtgtgcat actgtgtagt tacatagccc ttccaattct
3001 gggtccattt gcactagcaa attaaaatat gctttgattc atacttaaac ctgaaagcag
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3241 ggctctaaga ttcactttga ggtggaactt aaaaccagtg tactgtatgt atgcattggt
3301 aatagctact tttgcttcat agcttcatac caacaaaata tatttattag aatagtatga
3361 aagtactgga ggagctgaaa gaaaaacacc caaggctggg cgtggtggca cacgcctgta
3421 atcccagcac tttgggaggc cgaggcaggt ggatcacctg aggttgggag ttggagacca
3481 gcttgaccaa catggagaaa ccccgtctct actaaaaata caaaattggc cgggcgtggt
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3781 attttgttaa aattccccag atgattcttg gtatgaacga ctatattata aattttaaga
3841 tgtacttaga aatccttaag acatctagcc ccgtctctaa tagacaacac atttatattg
3901 cagatattac tttttttca gtttatgacc aggtatttat gaaggactat tggcagggaa
3961 aatatgaata tgttaacttt agcttatggc atcaatttac taaggaacaa caggctcacc
4021 aactgatgtc aaacataaaa acccccacat cagtctgata cgatatggta ctactttgaa
4081 tctgttacta gtaccatctt gacagaggat acatgctccc aaaacgtttg ttaccacact
4141 taaaaatcac tgccatcatt aagcatcagt ttcaaaatta tagccattca tgatttactt
4201 tttccagatg actatcatta ttctagtcct ttgaatttgt aaggggaaaa aaaacaaaaa
4261 caaaaactta cgatgcactt ttctccagca catcagattt caaattgaaa attaaagaca
4321 tgctatggta atgcacttgc tagtactaca cactttgtac aacaaaaaac agaggcaaga
4381 aacaacggaa agagaaaagc cttccttttgt tggcccttaa actgagtcaa gatctgaaat
4441 gtagagatga tctctgacga tacctgtatg ttcttattgt gtaaataaaa ttgctggtat
4501 gaaatgacac taaagtttgt caaaaaatga attcttaact tttctcccag agaaagggag
4561 acaaaaggag ctttttaata cctaatctac tttggaacat aaccgtatag ag
//
```

1.2. AMINO ACID SEQUENCE OF DRP1

Amino acid sequence was obtained from UniProtKB (UniProt number: O00429):

```
        10          20          30          40          50          60
MEALIPVINK  LQDVFNTVGA  DIIQLPQIVV  VGTQSSGKSS  VLESLVGRDL  LPRGTGIVTR

        70          80          90          100         110         120
RPLILQLVHV  SQEDKRKTTG  EENGVEAEEW  GKFLHTKNKL  YTDFDEIRQE  IENETERISG

        130         140         150         160         170         180
NNKGVSPEPI  HLKIFSPNVV  NLTLVDLPGM  TKVPVGDQPK  DIELQIRELI  LRFISNPNSI

        190         200         210         220         230         240
ILAVTAANTD  MATSEALKIS  REVDPDGRRT  LAVITKLDLM  DAGTDAMDVL  MGRVIPVKLG

        250         260         270         280         290         300
IIGVVNRSQL  DINNKKSVTD  SIRDEYAFLQ  KKYPSLANRN  GTKYLARTLN  RLLMHHIRDC

        310         320         330         340         350         360
LPELKTRINV  LAAQYQSLLN  SYGEPVDDKS  ATLLQLITKF  ATEYCNTIEG  TAKYIETSEL

        370         380         390         400         410         420
CGGARICYIF  HETFGRTLES  VDPLGGLNTI  DILTAIRNAT  GPRPALFVPE  VSFELLVKRQ

        430         440         450         460         470         480
IKRLEEPSLR  CVELVHEEMQ  RIIQHCSNYS  TQELLRFPKL  HDAIVEVVTC  LLRKRLPVTN

        490         500         510         520         530         540
EMVHNLVAIE  LAYINTKHPD  FADACGLMNN  NIEEQRRNRL  ARELPSAVSR  DKSSKVPSAL

        550         560         570         580         590         600
APASQEPSPA  ASAEADGKLI  QDSRRETKNV  ASGGGGVGDG  VQEPTTGNWR  GMLKTSKAEE

        610         620         630         640         650         660
LLAEEKSKPI  PIMPASPQKG  HAVNLLDVPV  PVARKLSARE  QRDCEVIERL  IKSYFLIVRK

        670         680         690         700         710         720
NIQDSVPKAV  MHFLVNHVKD  TLQSELVGQL  YKSSLLDDLL  TESEDMAQRR  KEAADMLKAL

        730
QGASQIIAEI RETHLW
```

2. CLONING

2.1. PRIMERS

Primer sequences for site-directed mutagenesis were designed with the bioinformatic tool PrimerX (http://www.bioinformatics.org/primerx/) and obtained from Eurofins MWG Operon (100 µM).

DNM1 primers:

E 61:	
Ep 10f	5´ – CTGGTCATATGGCTAGTTTAGAAGATC – 3´
Ep 10r	5´- CTGGTCTCGAGCAGAATATTACTAATAAGGG – 3´

DRP1 primers:

Ep 6f	5´ – CTGGTCATATGGAGGCGCTAATTCCTGTC- 3´
Ep 6r	5´ – CTGGTCTCGAGCCAAAGATGAGTCTCCCG- 3´
Ep 6rStop	5´- CTGGTCTCGAGTCACCAAAGATGAGTCTC – 3´

Note: DRP1 primer Ep 6r was used for constructs E 65 and E 67 – E 68 that were cloned into the vector pET-21. DRP1 primer Ep 6rStop was used for construct E 64 that was cloned into the vector pHIS-Parallel2.

DRP1 fusion/mutation primers:

E 64:	
Ep 26f	5´-GGTGAACCCGTGGATGGATCAGGATCAGGATCAGGATCA -3´
Ep 26r	5´-ATCAGCTGCTTCTTTTGATCCTGATCCTGATCCTGATCC – 3´
E 65:	
Ep 27f	5´-GGTGAACCCGTGGATGGATCAGGATCAGGATCAGGATCA -3´
Ep 27r	5´- CCGAGCAGATAGTTTTGATCCTGATCCTGATCCTGATCC – 3´
E 67:	
Ep 28f	5´- GACATTTTGACTGACATTAGAAATGC -3´
Ep 28r	5´- GCATTTCTAATGTCAGTCAAAATGTC -3´
E 68:	
Ep 29f	5´- GTAACACTATTGAAGATACTGCAAAATATATTG -3´
Ep 29r	5´- CAATATATTTTGCAGTATCTTCAATAGTGTTAC -3´
E 69:	
Ep 30f	5´- GAGCTATGCGGTGATGCTAGAATTTG -3´
Ep 30r	5´- CAAATTCTAGCATCACCGCATAGCTC -3´

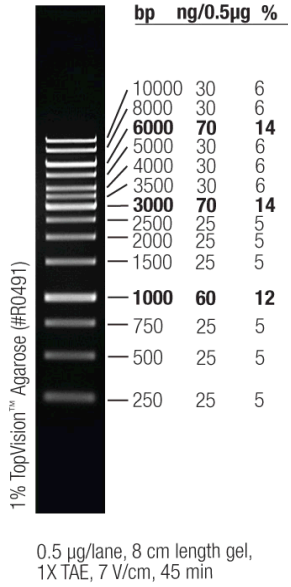
Primers flanking MCS of pET-21 and pHIS-Parallel2:

T7f	5´- TAA TAC GAC TCA CTA TAG GG – 3´
T7r	5´- CTA GTT ATT GCT CAG CGG T – 3´

2.2. MARKERS

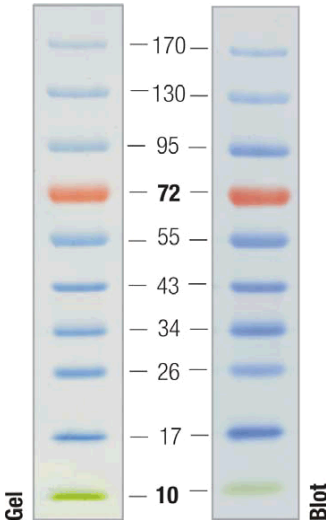
DNA GEL ELECTROPHORESIS

➢ 1 kb DNA Ladder *



SDS- POLYACRYLAMIDE GEL ELECTROPHORESIS

Prestained Protein Ladder*



* Both markers were obtained from Fermentas Life Sciences.

2.3. RESTRICTION SITES OF NDEI AND XHOI

NdeI:

5'...C A· T A T G...3'

3'...G T A T· A C...5'

and XhoI:

5'...C· T C G A G...3'

3'...G A G C T· C...5'

3. BUFFERS, REAGENTS AND ENZYMES

3.1. CLONING

3.1.1. ENZYMES

iProof High-Fidelity Polymerase (Bio-Rad)	0,04 u/µL
Fast digest restriction enzyme NdeI	1 u/µL
Fast digest restriction enzyme XhoI	1 u/µL
FastAP Thermosensitive Alkaline Phosphatase	1 u/µL
T4 DNA Ligase	5 u/µL
DreamTaq Green DNA Polymerase	5 u/µL
RNase-free DNase I	1 u/µL

Unless otherwise stated, enzymes were obtained from Fermentas Life Sciences.

3.1.2. AGAROSE GEL ELECTROPHORSIS

1% Agarose Gel	1 g Agarose	
	100 mL 1 x TAE	
50 x TAE Buffer	2 M Tris	242 g Tris
	5,7 % (v/v) Acetic acid	57,1 mL Acetic acid
	64 mM EDTA	18,6 g EDTA
	dd H_2O up to 1 L	
10 x DNA Loading Dye	2 mL 50 x TAE	
	30% Glycerol	
	trace of Bromphenolblue	
60 x GelRed	GelRed nucleic acid stain (Biotium Inc.) 10 000x	
	3 µL GelRed to 497 µL dd H_2O	
	final concentration: 3 x	

3.2. PROTEIN EXPRESSION

Ampicillin
final concentration: 50 µg/mL
50 mg/mL stock: 500 mg in 10 mL dd H_2O

IPTG
Isopropyl-ß-D-thiogalactopyranosid
final concentration: 0,42 mM
0,42 M stock: 1 g to 10 mL dd H_2O

PMSF
Phenylmethylsulfonylfluoride
final concentration: 1 mM
200 mM stock: 70 mg in 2 mL 96% Ethanol

Lysis Buffer	50 mM KH_2PO_4	6,8 g KH_2PO_4
pH 7.8	300 mM NaCl	60 mL 5 M NaCl
	0,1 mg/mL Lysozyme	0,1 g Lysozyme
	10 % Glycerol	100 mL Glycerol
	dd H_2O up to 1 L	

3.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-Polyacrylamide Gel	15 % Resolving gel	4 % Stacking gel
1.5 M Tris pH 8.8	18 mL	-
0.5 M Tris pH 6.8	-	9 mL
20 % SDS	360 µL	180 µL
40 % Acrylamide	27 mL	3.6 mL
dd H_2O	22.9 mL	23.5 mL
10 % APS	504 µL	270 µL
TEMED	54.4 µL	27.2 µL
		+ Bromphenolblue

SDS-Running Buffer	383 mM Glycine	28,8 g Glycine
pH 8.3	50 mM Tris	6 g Tris
	0,1 % SDS	1 g SDS
	dd H_2O up to 1 L	
10 x SDS Loading	60 mM Tris	600 µL 1 M Tris
Buffer pH 6.8	0,1 M DTT	0,15 g DTT
	5 % SDS	0,5 g SDS
	10 % Glycerol	1 mL Glycerol
	trace of Bromphenolblue	
Staining Solution	50 % Ethanol	500 mL Ethanol
	10 % Acetic acid	100 mL Acetic acid
	0,1 % Coomassie Brilliant Blue	1 g Coomassie B.B.
	dd H_2O up to 1 L	
Destaining Solution	25 % Ethanol	250 mL Ethanol
	10 % Acetic acid	100 mL Acetic acid
	dd H_2O up to 1 L	

3.4. PROTEIN PURIFICATION

3.4.1. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Equilibration buffer	20 mM Tris	20 mL 1 M Tris
pH 7.8	300 mM NaCl	60 mL 5 M NaCl
	10 % Glycerol	100 mL Glycerol
	10 mM Imidazole	10 mL 1 M Imidazole
	dd H_2O up to 1 L	
Wash Buffer	20 mM Tris	20 mL 1 M Tris
pH 7.8	300 mM NaCl	60 mL 5 M NaCl
	10 % Glycerol	100 mL Glycerol
	40 mM Imidazole	40 mL 1 M Imidazole
	10 % Sucrose	100 g Sucrose
	dd H_2O up to 1 L	
Elution Buffer	25 mM Tris	25 mL Tris
pH 7.8	300 mM NaCl	60 mL 5 M NaCl
	500 mM Imidazole	500 mL 1 M Imidazole
	15 % Glycerol	150 mL Glycerol
	dd H_2O up to 1 L	

3.4.2. SIZE EXCLUSION CHROMATOGRAPHY

SEC Buffer	30 mM Tris	30 mL 1 M Tris
pH 7.8	100 mM NaCl	20 mL 5 M NaCl
	1 mM EDTA	2 mL 0,5 M EDTA
	2 mM DTT	0,308 g DTT
	dd H_2O up to 1 L	
	sterile filtered and degassed	

SEC Buffer 2	30 mM Tris	30 mL 1 M Tris
pH 7.8	150 mM NaCl	30 mL 5 M NaCl
	4 mM $MgCl_2$	4 mL 1 M $MgCl_2$
	dd H_2O up to 1 L	
	sterile filtered and degassed	

SEC Buffer 3	20 mM Tris	20 mL 1 M Tris
pH 8	150 mM NaCl	30 mL 5 M NaCl
	2 mM EGTA	760 g EGTA
	4 mM $MgCl_2$	4 mL 1 M $MgCl_2$
	1 mM DTT	154 mg DTT
	dd H_2O up to 1 L	
	sterile filtered and degassed	

3.5. THERMOFLUOR ASSAY

List of buffers tested in Thermofluor assay:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	No Salt
B	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	200mM NaCl
C	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	400mM NaCl
D	NaAc pH4.0	NaAc pH4.4	Citrate pH5.0	Citrate pH5.4	Cacodyl pH6.0	Cacodyl pH6.4	HEPES pH7.0	HEPES pH7.4	TRIS pH8.0	TRIS pH8.4	CAPSO pH 9.0	CAPO pH 9.4	1000 mM NaCl
E	10mM ATP	10mM ADP	10mM GTP	10mM GDP	10mM GTP-γS	2 mM DTT	10mM DTT	10mM NADH	2 mM TCEP	10mM TCEP			Additives
F	2 % glycerol	10% glycerol	20% glycerol	40 % glycerol	20mM **Phenol**	20mM DMSO	30mM NiCl	10mM Thyio cyanate	20mM TFA	20mM Urea			
G	30mM CaCl	30mM MgCl	30mM ZiCl	30mM FeCl	30mM KCl	30mM LiCl							
H	20mM Glycine	10mM Alanine	10mM Methio nine	10mM Serine	10mM Arg inine	20mM PEG 400	10mM D(+)-Glucose	10mM D-Galact ose	20mM n-Octyl-β-D-gluco-pyrano side	10mM n-Dodecyl β-D-malto side			

3.6. GTPASE ASSAY

GTPase Assay	150 mM NaCl	30 mL 5 M NaCl
Buffer pH 7	25 mM HEPES	50 mL 0,5 M HEPES
	25 mM PIPES	50 mL 0,5 M PIPES
	dd H_2O up to 1 L	
20 x Reaction Buffer	25 mM HEPES	50 mL 0,5 M HEPES
pH 7	25 mM PIPES	50 mL 0,5 M PIPES
	100 mM $MgCl_2$	100 mL 1 M $MgCl_2$
	20 mM PEP	3,36 g PEP
	150 mM KCl	30 mL 5 M KCl

3.7. MISCELLANEOUS

100 mM GDP guanosine 5´-diphosphate sodium salt*
M_r = 443,2 g/mol
44.32 mg per 1 mL ddH_2O
adjust pH to 7 with 0,5 M NaOH

100 mM GTP guanosine 5`-triphosphate sodium salt hydrate*
M_r = 523,18 g/mol
52.32 mg per 1 mL ddH_2O
adjust pH to 7 with 0,5 M NaOH

100 mM GMP-PCP guanosine 5`-[beta,gamma-imido]triphosphate sodium salt hydrate*
M_r = 529,93 g/mol
52.99 mg per 1 mL ddH_2O
adjust pH to 7 with 0,5 M NaOH

*obtained from Sigma-Aldrich Life Sciences

7. REFERENCES

[1] Chang, C.-R., Blackstone, C. *Dynamic regulation of mitochondrial fission through modification of dynamin-related protein Drp1.* **Ann. N.Y. Acad. Sci. 1201: 34-39 : s.n., 2010.**

[2] Hoppins, S., Lackner, L., Nunnari, J. *The machines that divide and fuse mitochondria.* **Annu. Rev. Biochem, 76: 751-780 : s.n., 2007.**

[3] Benard, G., Karbowski, M. *Mitochondrial fusion and division: regulation and role in cell viability.* **Semin Cell Dev Biol. : s.n., 2009.**

[4] Otera, H., Mihara, K. *Molecular mechanisms and physiologic functions of mitochondrial dynamics.* **J. Biochem. 149(3):241-251 : s.n., 2011.**

[5] Seo, A.Y., Joseph, A.-M., Dutta, D., Hwand, J.C.Y., Aris, J.P., Leeuwenburgh, C. *New insights into the role of mitochondria in aging: mitochondrial dynamics and more.* **Journal of Cell Science, 123, 2533-2542 : s.n., 2010.**

[6] Praefcke, G.J.K., McMahon, H.T. *The dynamin superfamily: Universal membrane tubulation and fission molecules?* **Nature, Vol 5, 133-147 : s.n., 2004.**

[7] Zhang, Y., Gao, X., Garavito, R.M. *Biochemical characterization of human dynamin-like protein 1.* **Journal of Biochemistry, 150(6):627-33: s.n., 2011.**

[8] Knott, A.B., Perkins, G., Schwarzenbacher, R., Bossy-Wetzel, E. *Mitochondrial fragmentation in neurodegeneration.* **Nature Reviews, Neuroscience Vol. 9 : s.n., 2008.**

[9] Zhu, P.P., Patterson, A., Stadler, J., Seeburg, D.P., Sheng, M., Blackstone, C. *Intra- and intermolecular domain interactions of the C-terminal GTPase effector domain of the multimeric dynamin-like GTPase Drp1.* **JBC, 279: 35867-35974 : s.n., 2004.**

[10] Otera, H., Wang, C., Clelnad, M.M., Setoguchi, K., Yokota, S., Youle, R.J., Mihara, K. *Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells.* **J Cell Biol. 191(6): 1141-58 : s.n., 2010.**

[11] Chang, C.-R., Manlandro, C.M., Arnoult, D., Stadler, J., Posey, A.E., Hill, R.B., Blackstone, C. *A lethal de novo mutation in the middle domain of the dynamin-related GTPase Drp 1 impairs higher order assembly and mitochondrial division.* **Journal of Biological Chemistry, Vol. 285, NO. 42, 32494-32503 : s.n., 2010.**

[12] Danino, D., Hinshaw, J.E. *Dynamin family of mechanoenzymes.* **Current Opinion in Cell Biology 13: 454-460 : s.n., 2001.**

[13] Bourne, H.R., Sanders, D.A., McCormick, F. *The GTPase superfamily: conserved structure and molecular mechanism.* **Nature, Vol 349: 10.01.1991 : s.n., 1991.**

[14] Zhang, P., Hinshaw, J.E. *Three-dimensional reconstruction of dynamin in the constricted state.* **Nat Cell Biol. 3, 922-926 : s.n., 2001.**

[15] Ingerman, E., Perkins, E.M., Marino, M., Mears, J.A., McCaffery, J.M., Hinshaw, J.E., Nunnari, J. *Dnm1 forms spirals that are structurally tailored to fit mitochondria.* **The Journal of Cell Biology 170, 1021 - 1027 : s.n., 2005.**

[16] Chappie, J.S., Acharya, S., Leonard, M., Schmid,, S.L., Dyda, F. **G domain dimerization controls dynamin´s assembly-stimulated GTPase activity. 2010.**

[17] Shin, H.-W., Takatsu, H., Mukai, H., Munekata, E., Murakami, K., Nakayama, K. *Intermolecular and interdomain interactions of a dynamin-related GTP-binding protein, Dnm1p/Vps1p-like protein.* JBC, 274: 2780-2785 : s.n., 1999.

[18] Faelber, K., Posor, ., Gao, S., Held, M., Roske, y., Schulze, D., Haucke, V., Noé, F., Daumke, O. *Crystal structure of nucleotide-free dynamin.* Nature 477, 556-560 : s.n., 2011.

[19] Muhlberg, A.B., Warnock, D.E., Schmid, S.L. *Domain structure and intramolecular regulation of dynamin GTPase.* The EMBO Journal Vol. 16 No.22 pp. 6676-6683 : s.n., 1997.

[20] Zhang, Y., Chan, D.C. *New insights into mitochondrial fusion.* FEBS Lett. 581 (11):2168-2173 : s.n., 2007.

[21] Niemann, H.H., Knetsch, M.L.W., Scherer, A., Manstein, D.J., Kull, F.J. *Crystal structure of a dynamin GTPase domain in both nucleotide-free and GDP-bound forms.* The EMBO Journal Vol.20 No.21 pp. 5813-5821 : s.n., 2001.

[22] Reubold, T.F., Eschenburg, S., Becker, A., Leonard, M., Schmid, S.L., Vallee, R.B., Kull, F.J., Manstein, D.J. *Crystal structure of the GTPase domain of rat dynamin 1.* PNAS Vol. 102 No. 37: 13093-13098 : s.n., 2005.

[23] Gao, S., von der Malsburg, A.,Paeschke, S., Behlke, J., Haller, O., Kochs, G., Daumke, O. *Structural basis of oligomerization in the stalk region of dynamin-like MxA.* Nature, Vol. 465, doi:10.1038/nature08972 : s.n., 2010.

[24] Low, H.H., Löwe, J. *A bacterial dynamin like protein.* Nature Letter Vol. 444 doi:10.1038/nature05312 : s.n., 2006.

[25] Prakash, B., Renault, L., Praefcke, G.J.K., Herrmann, C., Wittinghofer, A. *Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism.* **The EMBO Journal Vol.19 No.17 pp. 4555-4564 : s.n., 2000.**

[26] Chappie, J.S., Mears, J.A., Fang, S., Leonard, M., Schmid, S.L., Milligan, R.A., Hinshaw, J.E., Dyda, F. *A pseudoatomic model of the dynamin polymer identifies a hydrolysis-dependent powerstroke.* **Cell 147, 209-222 : s.n., 2011.**

[27]. Ford, M.G.J., Jenni, S., Nunnari, J. *The crystal structure of dynamin.* **Nature, Vol 477, 561-566 : s.n., 2011.**

[28] Chen, X., Xu, X., Sun, Y., Zhou, J., Ma, Y., Yan, L., Lou, Z. *Purification, crystallization and preliminary X-ray crystallographic analysis of Arabidopsis thaliana dynamin-related protein 1A GTPase-GED fusion protein.* **Acta Crystallographica, F 68, 69-72 : s.n., 2012.**

[29] Bossy-Wetzel, E., Schwarzenbacher, R., Lipton, S.A. *Molecular pathways to neurodegeneration.* **Nature Medicine, Vol.10, pages S2-S9 : s.n., 2004.**

[30] Song, W., Chen, J., Petrilli, A., Liot, G., Klinglmayr, E., Zhou, y., Poquiz, P., Tjong, J., Pouladi, M.A., Hayden, M.R., Masliah, E., Ellisman, M., Rouiller, I., Schwarzenbacher, R., Bossy, B., Perkins, G., Bossy-Wetzel, E. *Mutant huntingtin binds the mitochondrial fission GTPase DRP1 and increases its enzymatic activity.* **Nat Med. 17(3): 377-382 : s.n., 2011.**

[31] Ingerman, E., Nunnari, J. *A continuous, regenerative coupled GTPase assay for dynamin-related proteins.* **Methods Enzymol. 404: 611-9 : s.n., 2005.**

[32] Bergfors, T. *Seeds to crystals.* **Journal of Structural Biology 142, 66-76 : s.n., 2003.**

[33] Walter, T.S., Mancini, E. J., Kadlec, J., Graham, S.C., Assenberg, R., Ren, J., Sainsbury, S., Owens, R. J., Stuart, D. I., Grimes, J. M., Harlos, K. *Semi-automated microseeding of nanolitre crystallization experiments.* Acta Cryst. F64, 14-18 : s.n., 2007.

[34] Borgstahl, G.E.O. *How to use dynamic light scattering to improve the likelihood of growing macromolecular crystals.* Methods in Molecular Biology, Vol. 363: Macromolecular Crystallography Protocols: Volume 1 : s.n.

[35] Niesen, F.H., Koch, A., Lenski, U., Harttig, U., Roske, Y., Heinemann, U., Hofmann, K.P. *An approach to quality management in structural biology: Biophysical selection of proteins for successful crystallization.* Journal of Structural Biology 162: 451-459 : s.n., 2008.

[36] Yoshida, H., Hensgens, C.M.H., van der Laan, J.M., Sutherland, J.D., Hart, D. J., Dijkstra, B.W. *An approach to prevent aggregation during purification and crystallization of wild type acyl coenzyme A: Isopenicillin N acyltransferase from Penicillium chrysogenum.* Protein Expression and Purification 41: 61-67 : s.n., 2005.

[37] Benvenuti, M., Mangani, S. *Crystrallization of soluble proteins in vapor diffusion for X-ray crystallography.* Nature Protocols, Vol.2 No.7, 1633-1651 : s.n., 2007.

[38] Kimber, M.S., Vallee, F., Houston, S., Necakov, A., Skarina, T., Evdokimova, E., Beasley, S., Christendat, D., Savchenko, A., Arrowsmith, C. H., Vedadi, M., Gerstein, M., Edwards, A. M. *Data mining crystallization databases: Knowledge-based approaches to optimize protein crystal screens.* Proteins: Structure, Function, and Genetics 51: 562-568 : s.n., 2003.

[39] DeLucas, L.J., Bray, T.L., Nagy, L., McCombs, D., Chernov, N., Hamrick, D., Cosenza, L., Belgovskiy, A., Stoops, B., Chait, A. *Efficient protein crystallization.* Journal of Structural Biology 142: 188-206 : s.n., 2003.

[40] Yamanaka, M., Inaka, K., Furubayashi, N., Matsushima, M., Takashi, S., Tanaka, H., Sano, S., Sato, M., Kobayashi, T., Tanaka, T. *Optimization of salt concentration in PEG-based crystallization solutions.* **L. Synchrotron Rad. 18, 84-87 : s.n., 2011.**

[41] Luft, J. R., Wolfley, J. R., Said, M. I., Nagel, R. M., Lauricella, A., Smith, J. L., Thayer, M. H., Veatch, C. K., Snell, E. H., Malkowski, M. G., Detitta, G. T. *Efficient optimization of crystallization conditions by manipulation of drop volume ratio and temperature.* **Protein Science, 16: 715 - 722 : s.n., 2006.**

[42] Guan, R.-J., Wang, M., Liu, X.-Q., Wang, D.-C. *Optimization of soluble protein crystallization with detergens.* **Journal of crystal growth 231: 273-279 : s.n., 2001.**

[43] Low, H.H., Sachse, C., Amos, L.A., Löwe, J. *Structure of a bacterial dynamin-like protein lipid tube provides a mechanism for assembly and membrane curving.* **Cell, 139(7): 1342-1352 : s.n., 2009.**

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