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Biochemical and Chemical Biology Approaches to Investigate and Target the Mitochondrial GTPase OPA1

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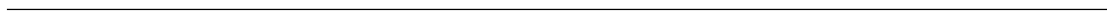
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Summary

Mitochondria are double membrane organelles of prokaryotic origin that are historically associated with cellular respiration, being the main site of ATP production through oxidative phosphorylation. Apart from this function, mitochondria are also crucial participants in mediating intrinsic apoptosis as host of numerous apoptogenic factors such as cytochrome *c*. These are stored within the cristae of mitochondria, which are sac-like structures that are formed by the inner mitochondrial membrane that are connected to the periphery of the membrane by the narrow tubular cristae junctions. Intact, cristae keep apoptogenic within the mitochondria. The disruption of cristae junctions and cristae remodeling allows contained molecules, including cytochrome *c*, to gain access to cellular cytosol and to initiate a cascade resulting in cell death.

Numerous mechanisms control cristae structure, among them being the large GTPase OPA1 (Optic atrophy protein 1). OPA1 forms complexes at the site of cristae junctions to maintain narrow openings and thus prevent efflux of cristae contents. During apoptosis, OPA1 oligomers are disrupted, coinciding with remodeling of the cristae, opening of their junctions and the release of apoptogenic factors. The role of OPA1 in this process was affirmed by previous data showing that the absence of OPA1, and its oligomers facilitate the induction of intrinsic apoptosis. Conversely, increased levels of OPA1 reduce cristae width and raise the threshold for inducing apoptosis. Through its control of cristae morphology, OPA1 also regulates mitochondrial metabolism by stabilizing the respiratory chain supercomplexes that reside on the cristae membranes.

Apart from the aforementioned functions, OPA1 mediates mitochondrial fusion together with the outer mitochondrial membrane protein Mitofusin1. Mitochondrial fusion and fission are crucial because they allow these highly dynamic organelles to rapidly adapt

to cellular demands and challenges. The machineries driving fusion and fission are located at the inner and outer mitochondrial membrane. The enzymes driving these membrane modulations are members of the family of large GTPases, which comprises dynamin-like proteins that commonly drive membrane fusion or fission in a GTP- and oligomerization-dependent manner.

The physiological importance of OPA1 is underlined by the fact that mutant forms of it are associated with the development of autosomal dominant optic atrophy (ADOA) in humans, the leading cause for inherited blindness worldwide originating from the selective death of retinal ganglion cells (RGCs). Interestingly, protein domains that are associated with the OPA1 GTPase domain and GTPase effector domain are hotspots for ADOA-associated mutations. The GTPase effector domain (GED) is thought to function similarly to the classic guanine nucleotide exchange factor of small GTPases to support GTP hydrolysis.

The aim of this thesis was to investigate the impact of mutations that are associated with the development of ADOA on the function of OPA1 through genetic, biochemical, and chemical biology approaches. First, we established a yeast-based assay to characterize OPA1 mutations by expressing different disease variants of OPA1 in a yeast strain lacking Mgm1, the yeast homologue of OPA1. Growth on a non-fermentable carbon source (glycerol) forces yeast cells to rely on respiration for energy production, an Mgm1-dependent process. Expression of an Mgm1-OPA1 chimeric protein restores the growth defect observed in the absence of Mgm1. After introducing ADOA-associated OPA1 mutations into the Mgm1-OPA1 fusion protein, we can assess the extent to which growth on a non-fermentable carbon source is rescued and determine their impact on the OPA1 function in this process. To complement this genetic approach, we developed an *in vitro* system to characterize the enzyme kinetics of wild type and mutant OPA1. To do so, we generated a protocol for the purification of recombinant OPA1 (rOPA1) using an inducible bacterial expression system and affinity based purification. rOPA1 was used to elucidate the enzymatic kinetics of OPA1, which shows positive cooperative hydrolysis behavior. Further biochemical characterization

suggests that the cooperative behavior is due to OPA1 oligomerization.

We used both systems to analyze the impact of mutations that are associated with ADOA classic and ADOA plus, a more severe multi-systemic form of the disease, in the GTPase domain and GED. Combining the data of both approaches allowed us to correlate mutations associated with ADOA classic with a loss of function phenotype and the ones associated with ADOA plus with a dominant negative effect on the wild type protein *in vivo*. Based on the kinetics data we obtained for the individual mutant proteins this effect is likely due to changes in the hydrolysis capacity and the cooperative kinetics of OPA1. While ADOA classic mutations lose their cooperative GTPase activity which correlates with a lower affinity for GTP, ADOA plus mutations maintain their cooperativity but have a severely altered catalytic turnover of GTP. Hence we hypothesize that retaining the cooperative behavior allows ADOA plus OPA1 variants to oligomerize with the wild type protein and thus impact negatively on their function. ADOA classic mutants however fail to cooperate with the wild type proteins. Similarly, co-expression of ADOA plus and wild type OPA1 in our yeast system revealed a dominant negative effect in respiration dependent growth and mitochondrial morphology while the ADOA classic mutations have no impact.

Together, these data suggests that ADOA-associated mutations can be biochemically grouped into ADOA classic and ADOA plus mutations based on their impact on the cooperative GTPase activity of OPA1.

We also sought to characterize the role of OPA1 in apoptosis. Given thatOPA1 has an anti-apoptotic role by controlling the release of cytochrome *c* from the intracristal space, we hypothesized that it inhibiting this function may yield a pharmacological drug to induce apoptosis in cancer cells. We made use of the rOPA1 to screen a library of small compounds. For that a semi-automated high-throughput screening (HTS) was set up in which the hydrolysis of GTP by rOPA1 served as readout to measure the inhibition of the GTPase activity of OPA1.

OPA1 activity was determined using a colorimetric assay that allows the measurement

of free organic phosphate that is released during the hydrolysis of GTP to GDP. Given that there is no existing inhibitor of OPA1 nor published model or structure of OPA1 to use in an *in silico* screen, we selected our HTS library based on diversity of compounds.

Our screen revealed 8 compounds that inhibit OPA1 GTPase activity in two repetitions of the screen. The compounds' effects were further confirmed in a dose-dependent response experiment, and also provided us with maximal inhibition (I_{max}), half maximal inhibition (IC_{50}), and Hill slope values for each. Based on their potency and biochemical effect on OPA1 we grouped them into potent and moderate inhibitors. Potent compounds inhibit OPA1 activity by more than 50% while moderate ones inhibit up to 50% of the activity. How the inhibitors impact on the different species of OPA1 will be focus of future experiments that may serve as scaffold for the identification of an OPA1 inhibitor *in vivo*.

In conclusion, the data presented in this doctoral thesis contributes to the field of ADOA research by correlating the mutations of ADOA classic and ADOA plus to functionally distinguished alteration on OPA1 GTPase function. Moreover, we developed a robust assay that can be used to characterize OPA1 disease mutation and phenotypes. Lastly, we set up and implemented a high-throughput screening that identified novel inhibitors of OPA1 GTPase activity *in vitro*.

Riassunto Dell'Attività Svolta

I mitocondri sono organuli citoplasmatici circondati da una doppia membrana deputati alla respirazione cellulare. Oltre alla produzione di ATP attraverso la fosforilazione ossidativa, i mitocondri hanno un ruolo chiave nella regolazione dell'apoptosi essendo sede di accumulo di molti fattori che controllano i meccanismi di morte cellulare, come il citocromo c. I fattori apoptogenici sono contenuti all'interno di estroflessioni della membrana mitocondriale interna che costituiscono veri e propri compartimenti chiamati creste mitocondriali. Il rimodellamento delle creste controlla l'apertura delle stesse a livello di sottili connessioni dette giunzioni delle creste, con conseguente rilascio dei fattori apoptogenici. Un regolatore chiave di questi processi è OPA1, una proteina con un grande dominio GTPasico. In condizioni normali OPA1 forma complessi oligomerici a livello delle giunzioni delle creste che sono in grado di chiudere le creste tenendo sequestrato il citocromo c, ma in seguito a stimoli apoptotici gli oligomeri vengono destabilizzati consentendo l'apertura delle giunzioni e il rilascio dei fattori apoptogenici. Il ruolo di OPA1 nell'apoptosi è supportato da precedenti evidenze sperimentali che dimostrano che l'assenza di oligomeri di OPA1 favorisce l'induzione di apoptosi e al contrario l'aumento dei livelli di OPA1 riduce l'ampiezza delle creste ritardando l'innesco dei processi di morte cellulare. L'importanza di OPA1 nel controllo della morfologia mitocondriale è essenziale, inoltre, per la corretta formazione dei supercomplessi della catena respiratoria che risiedono nella membrana interna del mitocondrio e che regolano l'efficienza respiratoria.

Infine è noto che OPA1 promuove la fusione mitocondriale cooperando con una proteina localizzata nella membrana mitocondriale esterna, Mitofusina 1 (MFN1). I mitocondri sono organelli molto versatili che, grazie ad eventi di fusione e fissione, sono in grado di modificare la propria struttura e morfologia a seconda delle condizioni cellulari. Le proteine che regolano questi eventi, localizzate sulle membrane esterna e interna del mitocondrio, appartengono alla famiglia delle dinamine. L'importanza fisiologica di OPA1

emerge dal fatto che mutazioni nel gene Opa1 causano l'atrofia dominante ottica (ADOA), la più frequente neuropatia ottica ereditaria, caratterizzata da progressiva riduzione dell'acuità visiva e atrofia temporale bilaterale del nervo ottico. I punti caldi di mutazione associati a questa patologia risiedono nel dominio GTPasico e nel dominio GED che si pensa abbia la funzione di idrolizzare il GTP in modo analogo ai fattori di scambio GTP-GDT (GEF).

Lo scopo di questa tesi era quello di approfondire le conoscenze sulla funzione di Opa1 mediante approcci genetici e biochimici. In particolare è stato studiato l'effetto di mutazioni associate ad ADOA sulla funzionalità di Opa1 attraverso l'utilizzo di saggi in lievito. In un ceppo di lievito privo dell'omologo di Opa1, Mgm1, sono state espresse diverse varianti-malattia di Opa1. I lieviti sono stati fatti crescere in un terreno con fonte di carbonio non fermentabile (glicerolo) che forza i mitocondri a respirare attivamente e questa capacità di ricavare energia dalla respirazione richiede la funzionalità di Mgm1. L'espressione nel ceppo privo di Mgm1 di una forma ibrida Mgm1-Opa1 recupera il difetto di crescita e consente di analizzare l'effetto di differenti mutazioni nel gene Opa1. Parallelamente è stato messo a punto un sistema *in vitro* per studiare la cinetica delle forme mutanti rispetto alla forma funzionale di Opa1. E' stato ottimizzato un protocollo per la purificazione di una forma ricombinante di Opa1 (rOpa1) che corrisponde alla forma solubile di Opa1 con un tag di istidine in posizione C-terminale che consentono la purificazione in colonna di affinità. Lo studio della cinetica enzimatica di questa proteina purificata ha consentito di dimostrare che la capacità di Opa1 di interagire è determinata primariamente dalla formazione di dimeri e quindi di tetrameri.

Entrambi questi approcci sono stati utilizzati per studiare l'effetto di differenti mutazioni nel dominio GTPase e nel dominio GED associate a ADOA e ADOA plus, una forma più grave e sistemica della malattia, E' stato osservato che mutazioni che causano la forma classica di ADOA determinano la perdita di funzione di Opa1 e mutazioni che determinano la forma sistemica di ADOA hanno un effetto dominante negativo sulla proteina funzionale. I dati di cinetica ci hanno permesso di identificare il difetto nella capacità di idrolisi e nella cinetica di interazione di Opa1. Mutazioni ADOA determinano una perdita di attività GTPasica

che correla con una ridotta affinità per il GTP. Mutazioni ADOA plus mantengono invece questa funzionalità ma modificano lo scambio catalitico di GTP. Di conseguenza è possibile ipotizzare che mutanti ADOA non perdano la capacità di formare oligomeri con la forma funzionale della proteina ma ne impediscano la normale attività, mentre mutanti ADOA siano incapaci di oligomerizzare. Infatti mutanti ADOA plus agiscono come dominanti negativi sull'attività respiratoria e sulla morfologia mitochondriale quando co-espressi in lievito con la forma wild type mentre mutanti ADOA non hanno alcun effetto su questi parametri. I dati ottenuti nel nostro sistema sperimentale in lievito conducono alle stesse conclusioni.

Questi risultati suggeriscono che mutazioni ADOA possano essere raggruppate sotto un aspetto biochimico sulla base del loro effetto sull'attività GTPasica. In generale inoltre è possibile concludere che questo saggio sperimentale costituisce un efficace strumento per valutare l'effetto di mutazioni *in vivo* e potrà essere utilizzato anche per analizzare l'impatto di nuove mutazioni di Opa1 sulla funzionalità della proteina

Infine abbiamo studiato il ruolo di Opa1 nell'apoptosi allo scopo di identificare composti in grado di inibire l'attività di Opa1 come fattore atiapoptotico che favorisce il trattenimento del citocromo c nelle creste mitocondriali. La scoperta di tali composti potrebbe essere cruciale per mettere a punto nuove strategie per combattere il cancro. La proteina ricombinante rOpa1 purificata è stata utilizzata per misurare la sua capacità di idrolizzare GTP in presenza di diversi composti chimici. L'obiettivo era quello di identificare il maggior numero di composti in grado di inibire l'attività GTPasica di Opa1 misurando mediante saggio colorimetrico il rilascio di fosfato nella reazione di idrolisi GTP-GDP. Questo tipo di analisi era particolarmente importante visto che fino ad ora nessun composto era stato identificato come specifico inibitore di Opa1 e neppure nessun modello in silico della struttura di Opa1 era stato generato per poter valutare con modelli predittivi la possibile azione inibitoria di composti chimici.

Dopo aver ripetuto due volte l'analisi, sono state identificate 8 sostanze in grado di inibire l'attività GTPasica di Opa1. Sulla base di specifici parametri questi composti sono stati

suddivisi in due categorie: potenti inibitori quando l'attività di Opa1 è ridotta più del 50% e medi inibitori (riduzione dell'attività di Opa1 inferiore al 50%). Il fatto che non sia mai stata osservata un'inibizione totale dell'attività di Opa1 e che il coefficiente di Hill non raggiunga mai velori pari a 1 suggerisce che ci siano più enzimi con effetto ridondante sull'attività di Opa1. In particolare i nostri dati indicano che rOpa1 può assumere diverse conformazioni con relative a e specifica attività GTPasica. Esperimenti futuri saranno mirati a determinare come i diversi inibitori agiscono sulle differenti forme conformazionali di Opa1. Inoltre determineremo se questi composti hanno attività inibitoria su Opa1 anche *in vivo*.

Introduction

1.1. Mitochondria

Mitochondria are cellular organelles that evolved from an ancient endosymbiotic event (Andersson and Kurland, 1999; Gabaldón and Huynen, 2004; Gray et al., 1999): hence, their proteins derive from both, old bacterial origin and new eukaryotic origin (Gabaldón and Huynen, 2004) and their composition varies within and between species in response to cellular and tissue-specific needs. This unique origin of the mitochondrion gives rise to distinct characteristics such as an own DNA, a unique double membrane and ultrastructural organization. Apart from being a major site for energy conversion, mitochondria participate in a plethora of important physiological processes making them essential components of most eukaryotic cells.

A unique characteristic of mitochondria that distinguishes it from other organelles is the presence of a genome. The human mitochondrial genome encodes for 13 proteins, 22 transfer RNAs and 2 mitochondrial ribosome-coding RNAs to compose a mitochondrial translation apparatus. A single cell contains numerous mitochondrial genomes (a situation known as "heteroplasmy") that are grouped into protein-rich complexes called nucleoids, which contain 1-8 genome copies (Schon and Area-Gomez, 2010). The majority of mitochondrial proteins however are encoded by the nuclear DNA, which after cytosolic translation are embedded into mitochondria through a complex import and sorting mechanism to reach the destined mitochondrial sub-compartments.

The mtDNA encoded proteins are core subunits of the mitochondrial respiratory complexes I, III, IV, and V which together with the entirely nuclear encoded complex II mediate ATP production through oxidative phosphorylation. Oxidative phosphorylation

(OXPHOS) produces most of the ATP in eukaryotic cells. The location of the respiratory chain complexes in the mitochondrial inner mitochondrial membrane (IMM) as well as the Krebs cycle in their matrix makes mitochondria essential sites of ATP production. The OXPHOS system comprises the electron transport chain (ETC) and the phosphorylation system of the ATP synthase. The ETC involves the reduction of O_2 to H_2O with electrons donated in a stepwise process by NADH and $FADH_2$ that are reduced using electrons extracted from organic intermediates by the Krebs cycle. The multiprotein complexes of the respiratory chain serve as electron carriers. Complex I (NADH dehydrogenase) catalyzes the transfer of electrons from NADH to CoQ. Complex II (succinate dehydrogenase) transfers electrons directly from succinate to CoQ. Electrons are transferred by complex III (ubiquinone-cytochrome c reductase) from reduced CoQ to cytochrome c, which in turn shuttles them to complex IV (cytochrome c oxidase). Complex IV finally catalyzes the electron transfer from cytochrome *c* to O_2. Through the coupled transfer of electrons to oxygen and the transport of protons by complex I, II, and IV from the matrix across the inner membrane into the space between outer and inner mitochondrial membrane, an electrochemical gradient is generated. The electrochemical gradient powers the terminal complex V of the chain, the ATP synthase, which catalyzes the synthesis of most cellular ATP. All oxidative steps in the degradation of carbohydrates, fats and amino acids converge at this final stage of cellular synthesis of ATP. To allow efficient aerobic respiration respiratory chain complexes assemble into higher ordered supercomplexes to enable efficient substrate channeling (Wittig et al., 2006). Their assembly however is dynamic allowing substrate entrance at different sites (Lapuente-Brun et al., 2013).

Apart from energy conversion, mitochondria participate in a multitude of physiological functions including calcium buffering, lipid synthesis, iron-sulfur (Fe-S) cluster biogenesis and thermogenesis. Moreover, mitochondria sense and react to cellular stress. In response to cellular stress mitochondria regulate and mediate quality control mechanisms such as autophagy (Gomes et al., 2011; Rambold et al., 2011) and programmed cell death (Tait and

Green, 2012).

Considering their participation in numerous cellular events, it is not surprising that dysfunctional mitochondria are considered as source for multiple human diseases including neurodegenerative disorders, cancer and the aging process.

Their numerous functions are also reflected in their versatile and complex structure (Frey and Mannella, 2000). Their double membrane compartmentalizes them, separates them from their surrounding and is the site of numerous cellular processes. In response to external cues mitochondria dynamically undergo morphological changes. Fragmentation or fission of the entire mitochondrial network may occur and mitochondrial ultrastructure may change dramatically. Their dynamic nature supports their highly adaptive behavior to diverse environmental conditions of different energy demands tightly linking function and structure. The efficiency of the respiratory chain for example is closely linked with the shape of cristae, which determines the biogenesis of respiratory chain supercomplexes (RCS) and serves to maintain optimal mitochondrial respiration (Cogliati et al., 2013). Loss of cristae structure results in less assembled supercomplexes and OXPHOS with lower efficiency as supercomplexes are lost and ATP dimer rows dissociate.

1.2. Mitochondrial Ultrastructure

Mitochondria have a distinct ultrastructural organization that is unique in eukaryotic cells. Unlike other cellular organelles, mitochondria possess a double membrane that creates four distinct compartments: the outer membrane (OM), the inner membrane (IM), the intermembrane space (IMS) and the mitochondrial matrix. The compartments contain different proteins and lipids which defines them as sites of distinct functions (Mannella et al., 1994).

Mitochondrial versatility is reflected in the dynamic organization of these

subcompartments. As early as in the 1960s mitochondrial ultrastructure was described to depend on the metabolic state of the cell resulting either in condensed or orthodox mitochondria (Hackenbrock, 1966). In the condensed state mitochondria have a contracted matrix compartment with wide cristae. In the orthodox state, the basal state in most tissues apart from muscles (Perkins et al., 2003; Schlame et al., 1999) the matrix space is expanded, thus mitochondria are less dense and cristae are contracted. To mediate morphological changes the IMM must undergoes structural rearrangements.

The outer mitochondrial membrane (OMM) separates mitochondrial content from the cytosol and serves equally as barrier and as interaction platform for numerous cellular events. Due to the presence of numerous voltage-dependent anion channels (VDAC) it is permeable to small molecules (Mr <5000) and ions that move freely, yet it serves as a barrier for larger molecules and thus protects cells from harmful mitochondrial products, such as immunogenic mitochondrial DNA (mtDNA) and apoptogenic factors. Import of nuclear encoded proteins hence relies on an import machinery consisting of the translocase of outer membrane (TOM) complexes. As interaction platform with the cytosol the OMM, apart from microtubules and cytoskeletal filaments mediates the interplay mitochondria have with other organelles including endoplasmic reticulum (ER), ribosomes and nucleus.

Different from the OMM, the IMM is impermeable to most molecules and ions, including protons (H$^+$), which ensures energy conservation by maintenance of a transmembrane potential ($\Delta\psi_m$) in form of a proton gradient across the IMM. The generation of ATP by OXPHOS involves the generation of a proton-motive force (Δp) across the IMM as the respiratory chain complexes pump protons across the membrane subsequently needed to drive the ATP synthase. The proton-motive force comprises the transmembrane potential and a transmembrane pH gradient (ΔpH).

Hence, transporter molecules are in place to mediate the crossing of proteins and metabolic intermediates across the impermeable IMM. The translocase of the inner

membrane (TIM) complexes imports proteins driven by the transmembrane potential. It pulls the positively charged presequence of the to-be imported proteins towards the negatively charged matrix across the membrane (Chacinska et al., 2009). Metabolite carriers drive the import of metabolites and cofactors, like the adenine nucleotide translocase (ANT) (Klingenberg, 2008).

While the composition of the OMM resembles that of eukaryotic cells, the IMM has a unique lipid composition, similar to that of bacterial membranes. It contains a high percentage (about 20%) of the anionic phospholipid cardiolipin (Ardail et al., 1990) that is found exclusively in mitochondria and participates in their form and function. It regulates the curvature of the IMM cristae and is required for the activity of several mitochondrial processes including the stability of OXPHOS complexes (Pfeiffer et al., 2003).

The IMM forms an architectural complex structure as it is divided into three unique zones, the inner boundary membrane (IBM), the cristae junctions (CJs), and the cristae that differ in morphology and protein distribution (Mannella, 2006; Vogel et al., 2006). The IBM describes regions of close contact of the OMM and the IMM, which contain complexes that link these two membranes including the machinery for protein import. Moreover, these regions were suggested to participate in energy transfer from matrix to cytosol, apoptosis, and lipid transfer between OMM and IMM.

Cristae junctions are the boundary regions between cristae and IBM. They are narrow openings of 18-30 nm in diameter (Frey and Mannella, 2000), thought to constitute a barrier preventing the diffusion of intracristal contents into the IMS (Mannella et al., 1994) and therefore serving as a control point for transfer of solutes, metabolites and proteins. The junctions are connected with the inner boundary membrane and outer membrane though the multi-protein complex termed "mitochondrial contact site and cristae organization system" (MICOS) complex, which is considered to be a crucial regulator of IMM shape (Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011). Loss of components of this complexes leads to the loss of cristae junctions.

Cristae are invaginations in the IMM which are the major site of the ETC and energy

production in mitochondria (Gilkerson et al., 2003) as they house the respiratory chain complexes and the ATP synthase (Davies et al., 2012). Moreover, they bear proteins involved in iron-sulfur biogenesis, protein translocation and synthesis, and in mitochondrial nucleoid maintenance. These convolutions of the IMM provide a large surface area providing a scaffold for thousands of respiratory chain supercomplexes and ATP synthase molecules improving its efficiency compared to a non-convoluted membrane.

The mitochondrial matrix is a densely packed compartment containing the mtDNA and its transcription and translation machinery. Moreover, as site for most fuel oxidation pathways (except glycolysis, which takes place in the cytosol) it contains the pyruvate dehydrogenase complex and the enzymes of the Krebs cycle, parts of the fatty acid β-oxidation pathway and the pathways of amino acid oxidation.

1.2.1. Mitochondrial Lipid Homeostasis

Mitochondrial membranes have a well-defined protein and lipid composition, which are required for their functional integrity and spatial organization. Hence, the maintenance of the proper composition is crucial for mitochondrial integrity.

Apart from the aforementioned cardiolipin mitochondrial membranes mainly contain phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidylinositol (PI) (Osman et al., 2011). Although there may be variations among organisms, PE and PC are the most abundant phospholipids (Zinser and Daum, 1995). Interestingly, mitochondrial membranes have high proportions of the nonbilayer-forming lipids cardiolipin and PE. Both cardiolipin and PE are synthesized in the mitochondria. The diglycerophopholipid cardiolipin is synthesized at the matrix side of the IMM through a cascade of enzymes from ER-derived PA (Tatsuta et al., 2014). Similarly PE is generated in mitochondria by the IMS facing IMM phosphatidylserine decarboxylase (PSD), which converts ER-derived PS into PE (Osman et al., 2011). Since the ER is the

major site of lipid synthesis (Tatsuta et al., 2014) an extensive transfer between ER and mitochondria is in place requiring close contact between the two organelles.

1.2.2. Mitochondrial Contact Sites

Mitochondria are connected with other cellular organelles including peroxisomes (Schrader, 2006), lysosomes (Soubannier et al., 2012) and the ER (de Brito and Scorrano, 2008) of which the latter has received most attention. Contacts between mitochondria and ER at the level of the mitochondrion-associated membranes (MAMs) represent structural and functional microdomains. They are physically tethered by the dynamin like protein mitofusin 2 (MFN2) that is expressed on both ER and mitochondria and enriched in MAMs where it forms homotypic and heterotypic interactions with mitochondrial MFN2 or mitofusin 1 (MFN1) (de Brito and Scorrano, 2008). The interface of ER and mitochondria is likely to be controlled by more interacting proteins. Fission 1 (Fis1), B cell receptor associated protein 31 (BAP31) (Iwasawa et al., 2011) and phosphofurin acidic cluster sorting protein 2 (PACS-2) (Simmen et al., 2005) were identified to participate in the tethering as well as the modulators mitochondrial ubiquitin ligase (MITOL) (Sugiura et al., 2013) and trichoplein/mitostatin (Cerqua et al., 2010).

Moreover, MAMs are essential sites of calcium buffering to control cellular calcium homeostasis (Rizzuto et al., 1993; Rizzuto et al., 1998). They also serve as platform for other processes that involve both organelles such as lipid metabolism (Vance, 1990) and apoptosis (Szalai et al., 1999). Recently, ER was also demonstrated to wrap around mitochondria at sites of fission events prior to Dynamin-related protein 1 (DRP1) recruitment (Friedman et al., 2011) mediated by the ER protein inverted forming 2 (INF2) that promotes actin filament assembly, which is a required event for DRP1-mediated fission to occur (Korobova et al., 2013).

1.3. Dynamics of Mitochondria - Mechanisms and Functions

Mitochondrial morphology differs widely among different cell types with respect to size, number, and dynamics (Bereiter-Hahn and Voth, 1994; Zick et al., 2009b). Their variability depends on cell types, physiological state, and developmental stage (Kuznetsov et al., 2009). To ensure proper distribution and morphology, mitochondria are highly mobile organelles that constantly move along the cytoskeleton and undergo fusion and fission (Youle and Van Der Bliek, 2012). Distribution in the cell is necessary to bring mitochondria to sites in demand of energy or calcium buffering (Hoth et al., 1997) since diffusion of both ATP and calcium is limited in the cytoplasm. Moreover, the network's plasticity makes mitochondria highly adaptable to dysfunction and stress and is therefore crucial for functional integrity of mitochondria and cellular health (Baker et al., 2014).

1.3.1. Mitochondrial Fusion and Fission

The mitochondrial network consists of interconnected organelles that continuously divide and fuse. This dynamic behavior ensures inheritance of mitochondria, mitochondrial trafficking and the interexchange and distribution of mitochondrial contents including substrates, metabolites, lipids and proteins throughout the cell. Moreover, it is required to maintain mtDNA and respiratory capacity and to cope with different levels of stress (Van Der Bliek et al., 2013). Fusion-fission imbalance is associated with mitochondrial and cellular dysfunction (Chen et al., 2007). The importance of mitochondrial dynamics is highlighted by the severe diseases associated with fusion defects caused by mutations in the proteins mediating it (Chen and Chan, 2009), which are described later. As mitochondria are characterized by two distinct membranes, tightly regulated mechanism are in place to orchestrate effective simultaneous fusion and fission of both: the combined action of OMM and IMM specific dynamin like GTPases together with accessory proteins drive this process.

In mammalian cells, fission is mediated by the dynamin-related protein 1 (DRP1) and distinct accessory proteins, Mid 49, Mid51, and Mff (Elgass et al., 2013). Cytosolic DRP1 gets recruited to the mitochondria where it forms oligomeric rings that constrict both mitochondrial membranes.

Different from fission, fusion is mediated by membrane anchored GTPases, the OMM Mitofusin 1 (MFN1) and 2 (MFN2) and the IMM optic atrophy 1 (OPA1). They are dynamin like GTPases that tether and fuse the membrane they are associated with. Moreover, there is increasing evidence that mitochondrial shape is not independent of motility. The OMM MFN2 for example was shown to physically interact with the adaptor proteins Miro and Milton (Misko et al., 2010).

Elongating mitochondria via increased fusion and/or decreased fission is a mechanism to overcome low levels of stress such as starvation (Van Der Bliek et al., 2013). The inhibition of fission protects mitochondria from excessive autophagosomal degradation (Gomes et al., 2011; Rambold et al., 2011) like stress-induced mitochondrial hyperfusion ensures survival upon exposure to low levels of toxins (Tondera et al., 2009). Increasing fusion or decreasing fission is likely beneficial because damaged mitochondria can either be complemented by fusion through the exchange of mitochondrial contents with healthy counterparts (Van Der Bliek et al., 2013). The intermixing and compensation plays also a crucial role in overcoming mtDNA defects since mutated mtDNA may be compensated with wildtype DNA (Chen et al., 2005). Hence, mitochondrial fusion represents an important part of the mitochondrial quality control system.

Fragmentation of the mitochondrial network by decreased fusion and increased fission on the other hand occurs with high levels of stress (Van Der Bliek et al., 2013). The key inducer of fragmentation is mitochondrial dysfunction, for example when mitochondrial membrane potential is lost (Cereghetti et al., 2008; Ishihara et al., 2006; Ishihara et al., 2003). Fragmented and damaged mitochondria may subsequently be selectively removed

by autophagy to prevent accumulation of dysfunctional organelles (Parone et al., 2008). Also during apoptosis fission rates drastically increase (Frank et al., 2001b) contributing to the cell death program.

1.3.2. Mitochondrial Motility

The dynamic morphology of mitochondria not only relies on fission and fusion but also on active transport mechanisms that traffic mitochondria throughout the cell. Motor-adaptor complexes that are associated to the OMM surface mediate their transport. The multifunctional microtubule-dependent motor proteins dynein and kinesin-1 bind cytoskeletal filaments and use ATP hydrolysis to generate the required energy for transport. Anterograde transport away from the nucleus to their site of action is primarily driven by kinesin-1 and retrograde movement towards the soma of the cell is mediated by dyneins ((Hollenbeck and Saxton, 2005; Ligon and Steward, 2000). They bind to mitochondria through interactions with the proteins Miro and milton with Miro being anchored into the OMM and Milton serving as adaptor between Miro and the motor proteins (Schwarz, 2013). The intriguing hypothesis that mitochondria moving retrogradely may represent older or damaged mitochondria while anterograde populations comprise healthy mitochondria (Miller, 2004) is still under debate. The importance of mitochondrial motility is particularly evidenced in neurons since their long axons require efficient mitochondrial distribution to satisfy local energy demands and calcium buffering (Schwarz, 2013). In line with this, defects in mitochondrial trafficking are associated with numerous neurodegenerative diseases.

1.3.3. Mitophagy

Mitochondrial fusion and fission can partially rescue damaged mitochondria by diluting or segregating deleterious components. Severely damaged mitochondria however, due to energy deprivation, oxidative stress, or hypoxia are selectively removed by the autophagic

machinery to prevent cell death, a process referred to as mitophagy. It describes the selective sequestration of damaged mitochondria by autophagosomes and subsequent degradation in the lysosome (Elmore et al., 2001; Lemasters, 2005). Selective removal of damaged mitochondria is mediated through the PINK1/Parkin pathway. The ubiquitin ligase PINK1 stabilizes in mitochondria upon loss of membrane potential or the accumulation of misfolded proteins and translocates to the OMM (Narendra et al., 2010b). Subsequently, it phosphorylates ubiquitin (Kane et al., 2014; Koyano et al., 2014), Parkin (Kondapalli et al., 2012), and MFN2 (Chen and Dorn, 2013), which leads to the activation ubiquitin ligase parkin and induces a feed-forward amplification loop stimulating Parkin translocation to the mitochondria and Parkin-mediated ubiquitination of mitochondrial OMM proteins (Kane et al., 2014).

Numerous OMM proteins are targets of Parkin, including mitofusins, VDAC, TOM20, Miro, and Fis1 (Sarraf et al., 2013). As a result mitochondria loose their ability to fuse and their mobility, which isolates them from the remaining mitochondrial network. Ubiquitination induced degradation of mitochondrial proteins promotes the recruitment of ubiquitin-binding autophagy receptors such as p62 (Geisler et al., 2010). This in turn elicits the targeting of the damaged mitochondria to LC3-positive phagophores for clearance in the lysosome (Narendra et al., 2010a). Defects in the pathways mediating mitophagy result in harmful accumulation of dysfunctional mitochondria for which mitophagy plays a key role as defense mechanism and in cellular quality control process.

1.4. Mitochondria-Shaping Proteins

Proteins mediating the constant morphological rearrangement of the mitochondrial network and ultrastrucutre are numerous. Both, mitochondrial dynamics and ultrastructure are interlinked by the overlapping participation of some protein in both processes exemplified by the IMM GTPase OPA1 that mediates fusion of the IMM and simultaneously participates in cristae formation.

1.4.1. Proteins Shaping Mitochondrial Ultrastructure

Despite the importance of mitochondrial structural organization for organellar integrity and function, the components orchestrating the morphology of the inner membrane domains are still only partially understood. Apart from inner membrane fusion GTPase OPA1, whose role will be discussed in a later chapter, also respiratory supercomplexes, oligomers of the ATP synthase, lipid composition, scaffolding proteins and the mitochondrial contact site and cristae organization system (MICOS) complex have been proposed to determine the IMM shape. Although each of the mentioned proteins performs a non-redundant role within mitochondria they cooperatively determine mitochondrial ultrastructure.

The ATP synthase assembles into dimers that assemble into higher order orligomers, which unique structure is assumed to participate in membrane curvature at the tips of the cristae (Paumard et al., 2002; Strauss et al., 2008). Their loss by depletion of the dimer-specific subunits results in altered cristae morphology with extended onion-like structures (Paumard et al., 2002).

The formation of cristae junctions is mediated by the MICOS complex, which is considered to be a crucial regulator of IMM shape. It is embedded in the inner membrane with domains facing the IMS through which cristae membranes are linked to the IBM (Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011). Its six core subunits in yeast are Mic60, Mic10, Mic19, Mic27, Mic26, and Mic12 of which all have mammalian homologues except Mic12 (Alkhaja et al., 2012; An et al., 2012; Darshi et al., 2011; Head et al., 2011; Weber et al., 2013; Xie et al., 2007). Deletion of any of these subunits results in loss of CJs, stacked and lamellar IMMs, and in deficient respiratory growth (Barbot et al., 2015; Friedman et al., 2015). The MICOS complex is considered to impact on cristae architecture by interacting with the lipid environment, namely cardiolipin, and the respiratory complexes (Friedman et al., 2015). The common phenotype of deletions of single subunits of the complex suggests a cooperative shared function. Many of the subunits however have been ascribed distinct

independent functions from the MICOS complex, like mitofilin, the mammalian homologue of Mic60 interacts with the TOM complex and promotes protein import into the IMS (von der Malsburg et al., 2011) linking IMM architecture and function.

Prohibitins form large, multimeric ring complexes formed by multiple subunits in the IMM and are suggested to act as scaffolding proteins of the IMM (Merkwirth et al., 2008). Moreover, they indirectly impact on mitochondrial ultrastrucutre by participating in cardiolipin metabolism (Richter-Dennerlein et al., 2014), clustering the phospholipids cardiolipin and PE at distinct sites in the IMM and protein recruitment to these respective sites (Osman et al., 2009). Moreover, it impacts on the processing of the OPA1 (Merkwirth et al., 2008). Genetic depletion in mice leads to embryonic lethality (Carsten Merkwirth, 2012), while depletion at a cellular level indirectly results in aberrant cristae morphogenesis, impaired cell proliferation and cell death in an OPA1-dependent manner (Merkwirth et al., 2008).

Apart from protein-driven membrane morphology, also mitochondrial lipids, particularly cardiolipin contributes to the characteristic structure of the IMM. It modulates the dynamic behaviour of mitochondrial membranes and their supramoeclular organization. Indeed, the stability of several multiportein complexes in both mitochondrial membranes depens on cardiolipin (Pfeiffer et al., 2003). This is highlighted by a disease, Barth syndrome, which is a mitochondrial disorder caused by mutations in tafazzin, which is involved in the biosynthesis of mitochondrial lipids such as cardiolipin (Schlame and Ren, 2006). Mitochondria of Barth syndrome patient exhibit an 80% reduction in cardiolipin coinciding with severely ultrastructural change of the mitochondria (Schlame and Ren, 2006). Whether cardiolipin participates directly in the membrane curvature or indirectly through stabilizing protein complexes such as the respiratory chain supercomplexes is unclear however. Apart from the here-listed proteins, other proteins have been suggested to be required for cristae maintenance including Sam50 and adenonucelotide transporters (Patten et al., 2014) whose roles require further investigations however.

1.4.2. Proteins Mediating Fusion

Despite being tightly coordinated, OMM and IMM fusion are mechanistically distinct mechanisms (Legros et al., 2002; Meeusen et al., 2004; Song et al., 2007). Mitochondrial fusion is a two-step process with OMM fusion and IMM fusion being two separable events. MFN1 and MFN2 promote OMM fusion while IMM fusion is performed by OPA1. The mechanisms underlying the coordination of the two events are still an open question.

Mitofusin1 and 2

Mitofusin 1 and 2 display high (81%) similarity and localize both to the OMM (Legros et al., 2002; Santel et al., 2003; Santel and Fuller, 2001). Both are dynamin like GTPases and possess a GTPase domain, a stalk and a GTPase effector domain (GED)-like α-helix (Praefcke and McMahon, 2004). Their two coiled coil domains are involved in protein interactions while their U-shaped two transmembrane domains anchor the proteins into the OMM, with the C- and N-terminal ends facing the cytosol (Koshiba et al., 2004; Rojo et al., 2002). Both mitofusins participate in mitochondrial fusion yet they were assigned mechanistically distinct functions during the fusion process (Chen et al., 2003; Eura et al., 2003). Strikingly however, mouse models for either of the mitofusins or a double knockout mouse are embryonical lethal (Chen et al., 2003). Moreover, mutations in MFN2 render the protein inactive associate with Charcot-Marie-Tooth type IIA (CMTIIA), a peripheral sensorimotor neuropathy (Zuchner et al., 2004).

While MFN2 participates in numerous mitochondrial mechanisms in addition to fusion, MFN1 appears to predominantly participate in mitochondrial fusion. Although it primarily functions as tether of the two to-be fused mitochondria (Koshiba et al., 2004), the deletion of MFN2 results in a less severe mitochondrial phenotype than that of MFN1 (Chen et al., 2003). Moreover, MFN1 can compensate for the fusion defect observed in the absence of MFN2 while *vice versa* was not observed (Chen et al., 2003; Cipolat et al.,

2004). The fusogenic property of mitofusins relies on their GTPase activity. *In vitro* analyses demonstrated MFN1 to hydrolyse GTP more efficiently (Ishihara et al., 2004) providing an explanation for it being more fusogenic. MFN2 additionally plays a key role in Ca^{2+} signaling as it tethers mitochondria and ER at the protein rich mitochondria associated membranes (MAMs) (de Brito and Scorrano, 2008). A proline rich domain, a protein-protein interaction domain in MFN2 that is not present in MFN1 is a possible mediator of this (Kay et al., 2000). Moreover, MFN2 is an interconnection point of mitochondrial fusion and motility (Baloh et al., 2007) as it interacts with the mitochondrial adaptors of their transport machinery Miro and Milton (Misko et al., 2010).

Both MFN1 and MFN2 are regulated by protein modifications. The ubiquitin ligase Parkin targets mitofusins, which subsequently dictates their degradation by the proteasome (Ziviani et al., 2010). Moreover, also the Jun N-terminal kinase (JNK) phosphorylates MFN2 specifically in stress conditions, which targets it for degradation through the ubiquitin-proteasome pathway resulting in mitochondrial fragmentation and facilitated cell death (Leboucher et al., 2012). Apart from being a target for proteasomal degradation itself, phosphorylation of MFN2 by PINK1 is involved in Parkin recruitment to mitochondria, which mediates Parkin dependent mitophagy (Chen and Dorn, 2013). Moreover, oxidized glutathione (GSSG), a core cellular stress indicator, induces induce mitochondrial fusion by generation of a disulphide-mediated MFN2 homooligomer in *cis* that stimulates docking and subsequent fusion of opposing mitochondria (Shutt et al., 2012) linking mitochondrial dynamics and the intracellular redox state.

MFN1 has only recently been identified to be target of post-translational modifications. It is phosphorylated by the extracellular-signal-regulated kinase (ERK) at a site required for its role in mitochondrial fusion. Phosphorylation stimulates its interaction with the proapoptotic BCL-2 family member BAK resulting in its activation and eventually enhances cell death, while diminishing MFN1 homooligomerization, which is required for its fusogenic function (Pyakurel et al., 2015).

As OMM and IMM membrane fusion are distinct events, MFN1 cooperatively functions

with the IMM OPA1 to drive full fusion (Cipolat et al., 2004).

OPA1

Genetic ablation of OPA1 induces mitochondrial fragmentation owing to an unopposed division machinery (Cipolat et al., 2004; Griparic et al., 2004; Ishihara et al., 2006; Olichon et al., 2003; Song et al., 2009) whereas a mild overexpression promotes elongation (Cipolat et al., 2004). In monocytes isolated from ADOA patients the mitochondrial network appears disorganized, further supporting OPA1 role in mitochondrial fusion (Delettre et al., 2001). OPA1 was shown to promote the fusion of mitochondria in cooperation with the outer mitochondrial membrane protein MFN1 (Cipolat et al., 2004). Although they are tightly coordinated they are mechanistically distinct events (Legros et al., 2002; Meeusen et al., 2004) as shown by ongoing OMM fusion in cells devoid of OPA1 (Song et al., 2007). A detailed discussion on how OPA1 promotes IMM fusion follows in the chapter on OPA1.

1.4.3. Proteins Mediating Fission

Mitochondrial fission is also mediated by a large dynamin like GTPase, namely DRP1. Differently from MFNs and OPA1, DRP1 resides in the cytosol and requires recruitment to the mitochondria where it together with OMM accessory proteins mediates fission. No protein that specifically mediates IMM fission has been identified and DRP1-dependent constriction of the OMM is commonly considered to force constriction of the IMM as well, resulting in the simultaneous division of both membranes (Westermann, 2010b). Whether a soluble form of OPA1 may participate in fission as it was suggested based on its colocalization with fission sites (Anand et al., 2014) or the protein MTP18 whose overexpression results in fragmentation (Tondera et al., 2005) remains to be verified.

DRP1

DRP1 is a dynamin like GTPase that mediates mitochondrial fission in a GTP hydrolysis dependent manner. Its inhibition results in elongated mitochondria (Pitts et al., 1999; Smirnova et al., 1998). DRP1 mostly exists in cytosolic pools and only a fraction is found at mitochondria at the sites of constrictions (Labrousse et al., 1999; Smirnova et al., 2001). Translocation to the mitochondria and activation of DRP1 is tightly regulated by both posttranslational modification and its recruitment to the OMM through adaptor proteins. The importance of these regulatory steps in DRP1 activity is emphasized by the unaltered mitochondrial morphology upon overexpression of DRP1 (Smirnova et al., 1998). Mitochondrial dysfunction is a key trigger to induce DRP1 translocation to initiate mitochondrial fission (Ishihara et al., 2003).

While residing in the cytosol DRP1 forms a complex with calcineurin and cyclophilin A. Upon activation by an increase of cytosolic calcium following mitochondrial dysfunction, calcineurin dephosphorylates DRP1 at serine 637 inducing translocation and subsequent fission (Cereghetti et al., 2008). Calcineurin is counteracted by phosphorylation of DRP1 at the same residue by protein kinase A (PKA) (Cribbs and Strack, 2007) and calmodulin dependent protein kinase 1a (CAMK1a) (Chang and Blackstone, 2007). Strikingly, despite the same target amino acid, PKA phosphorylation leads to mitochondrial elongation and CAMK1a phosphorylation to mitochondrial fission through a yet unknown mechanism (Han et al., 2008). PKA mediated phosphorylation is important during the regulation of mitochondrial function in autophagy and disease. Upon induction of autophagy by starvation DRP1 phosphorylation by PKA protects mitochondria from mitophagy, which allows cell survival (Gomes et al., 2011)). The residue serine 637 is positioned at a site that mediates oligomerization, which is inhibited by its phosphorylation as demonstrated by its crystal structure suggesting an oligomerization dependent effect.

DRP1 is subjected to other posttranslational modifications. During mitosis for

example serine 616 gets phosphorylated by cyclin-dependent kinase 1 (Cdk1) to induce mitochondrial fission (Taguchi et al., 2007). The ubiquitin ligase MITOL targets DRP1 on the mitochondrial membrane for degradation (Karbowski et al., 2007) while sumoylation stabilizes it by protecting it from ubiquitin binding and subsequent degradation by the proteasome (Harder et al., 2004; Wasiak et al., 2007; Zunino et al., 2007). Once translocated to the mitochondria, DRP1 binds to its adaptors on the OMM.

Accessory Proteins of DRP1- Fis1, Mff and MiD49/51

Apart from protein modifications, OMM adaptor proteins participate in the control of DRP1 translocation to mitochondria. While Fis1 still has an unclear involvement in DRP1 recruitment, Mff, Mid49 and MiD51 were shown to directly recruit DRP1 to the OMM (Otera et al., 2010; Palmer et al., 2011). Moreover, recent research suggests that there are likely multiple pathways to recruit DRP1 depending on physiological cues (Losón et al., 2013; Palmer et al., 2011; Sesaki et al., 2014).

Human Fis1 (hFis1) is an integral OMM protein (James et al., 2003) whose overexpression results in mitochondrial fragmentation. As it does not possess any enzymatic activity it was postulated to stabilize effector proteins on the OMM. Accordingly, mitochondrial fragmentation by hFis1 overexpression can be blocked by expression of dominant negative mutants of DRP1 (James et al., 2003). The genetic depletion in yeast of the Fis1 homologue reduces DRP1 localization to the mitochondria and its crystal structure identified a loop that participation in DRP1-dependent fission. Despite the well-defined role in yeast, the involvement of hFis1 is still unclear. Studies demonstrating a direct interaction of hFis1 and DRP1 supporting a conserved role of hFis in DRP1-dependent fission from yeast to mammals (Yoon et al., 2003) and its overexpression results in mitochondrial fragmentation and perinuclear clustering resembling that of DRP1 overexpression (Stojanovski et al., 2004; Yoon et al., 2003; Yu et al., 2005). However, this was challenged by studies that show unaffected mitochondrial morphology in cells that lack hFis1 (Otera et al., 2010) and that

its depletion does not affect DRP1 recruitment (Lee et al., 2004). These contradictory observations may suggest that hFis1 participates in mitochondrial fission but is dispensable for DRP1-mediated fission to occur. Importantly, hFis1 is also involved in other processes; it induces ER-dependent apoptosis and participates in triggering mitophagy to remove damaged mitochondria (Alirol et al., 2006; Gomes and Scorrano, 2008).

The mitochondrial fission factor (Mff) seems to be the strongest candidate as DRP1 receptor. It is anchored into the OMM *via* its C-terminal transmembrane domain and is visualized as puncta *in vivo* (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). When ablated, mitochondrial translocation of DRP1 is inhibited resulting in impaired fission and causes mitochondrial elongation (Losón et al., 2013). Similarly, overexpression induces mitochondrial fission in a strictly DRP1-dependent manner (Otera and Mihara, 2012). The physical interaction of DRP1 and Mff was demonstrated *in vivo* and *in vitro*. In fact, in cells depleted of Mff DRP1 remains in the cytosol and does not get recruited to the mitochondria (Otera et al., 2010). A recent mouse model in which Mff was genetically ablated supports a role of Mff in Drp-dependen fission (Chen et al., 2015). In fact, reducing mitochondrial fusion by ablating MFN1, which is lethal by itself (Chen et al., 2007), rescues the phenotype of mice with a gene trap disruption of Mff (Mff[gt]) mice while MFN2 ablation does not (Chen et al., 2015). This strongly suggests, that the observed phenotype of Mff[gt] mice derives from unopposed fusion events and hence a role of Mff in mitochondrial fission.

MiD49 and Mid51 are also membrane-anchored proteins that face the cytosol. They form rings-like structures around mitochondria similar to DRP1 rings. Interfering with their expression reduces DRP1 recruitment to the OMM and subsequent fission resulting in a highly connected network (Losón et al., 2013). Although their double knockdown was shown to lead to mitochondrial elongation, overexpression of either of them also resulted in mitochondrial elongation (Losón et al., 2013), questioning whether and how they recruit DRP1 to mitochondria.

1.4.4. Evolutionary Considerations - Yeast versus Mammals

The basic tenets of mitochondrial dynamics have been discovered in yeast, a commonly used model organism for eukaryotic mechanisms. An increasing number of findings however suggest that there are numerous differences between yeast and mammalian cells. While many of the proteins mediating the processes in mammalian cells possess homologues in yeast, many of them have adopted additional and/or different roles and a growing number of proteins in yeast as well as mammalian cells do not possess homologues in the other organism.

Similarly to mammalian cells, fusion in yeast cells is mediated by an outer and an inner mitochondrial membrane GTPase, Fzo1 and Mgm1, which are homologous to Mitofusins and OPA1 (Hermann et al., 1998; Wong et al., 2000). They are considered the core components of the mitochondrial fusion machinery. A third protein participates in fusion in yeast: the outer membrane protein Ugo1 (Sesaki and Jensen, 2001), which connects and coordinates the outer and inner membrane fusion machineries by physically interacting with Fzo1 and Mgm1 (Sesaki and Jensen, 2004). The proposed mechanism is that Ugo1 acts as an Fzo1 effector to facilitate its assembly in a GTP-dependent manner to promote tethering of OMMs (Anton et al., 2011). Moreover, it seems to be involved in regulating the lipid composition of the IMM (Hoppins et al., 2009). Recently, a mammalian modified carrier protein, SLC25A46, has been identified that was suggested to functionally resemble Ugo1 (Abrams et al., 2015). It localizes to the OMM and interacts with the MICOS component Mic60/mitofilin. Strikingly, it was identified based on a genome-wide screen for mutations in ADOA and CMTIIA patients linking both neurological diseases genetically and functionally (Abrams et al., 2015).

The homologue of the fission driving GTPase of the mammalian DRP1 is Dnm1, which is required for mitochondrial fission (Otsuga et al., 1998; Smirnova et al., 1998). Its interaction partners on the OMM comprise Fis1 and Mdv1 (Mozdy et al., 2000; Tieu et al., 2002). The

cytosolic Mdv1 represents a linker protein that interacts with the membrane anchored Fis1 once recruited to the mitochondria and Dnm1 (Tieu et al., 2002). Moreover, it functions as a Dnm1 effector that mediates its GTP-dependent oligomerization and polymerization, which is a crucial step in Dnm1 mediated fission (Lackner et al., 2009). The fission complex harbors additional proteins including Caf4 (Griffin et al., 2005), Num1 (Dimmer et al., 2002) and Mdm36 (Hammermeister et al., 2010) that not only mediates fission but also motility within the cell similarly as the mammalian OMM fusion protein MFN2 was suggested to interact with the MITOL of the motility machinery. While human Fis1 has an unclear role in mammalian cells, the homologue in yeast efficiently functions as receptor for Dnm1. Hence, different from in mammalian cells its knockdown blocks Dnm1 translocation (James et al., 2003). As in mammals, mitochondria shaping proteins in yeast are subjected to numerous regulatory pathways. Different from the core machineries that drive fission and fusion their regulation is more diverse between mammalians and yeast (Westermann, 2010a). Fzo1 for example is degraded at a steady-state level through the ubiquitin-protease system that is required for mitochondrial integrity (Cohen et al., 2008).

The basic mitochondrial ultrastrucutre in yeast resembles that found in mammalian cells hence it is not surprising to find that the IMM architecture is determined by MICOS in both yeast and mammalian. As aforementioned MICOS was identified in yeast, yet mammalian homologues of most components have been identified.

Conversely, contact sites between mitochondria and ER are tethered by the multiprotein tether structure termed the ER-mitochondria encounter structure (ERMES) in yeast whose components are not conserved in mammals (Kornmann et al., 2009). Future investigations on proteins that mediated the ER-mitochondria tether will clarify if an ERMES resembling structure exists in mammalian cells.

1.5. Mitochondrial Dynamics as disease hub

Defect in the components of mitochondria shaping proteins primarily affect neuronal functions as nerve cells have a high-energy demand and strictly depend on mitochondrial function.

1.5.1 In Neurodegenerative Diseases

Mutations in mitochondrial fusion genes *OPA1* and *MFN2* cause neurodegeneration. Mutations in OPA1 are causative for autosomal dominant optic atrophy (ADOA), the most common form of inherited childhood blindness (Alexander et al., 2000; Delettre et al., 2000). Patients with ADOA experience progressive loss of vision due to degeneration of retinal ganglion cells, the axons of which form the optic nerve. Since this Thesis focuses on the biochemical properties of OPA1 in ADOA, we will discuss below the disease in greater detail.

Mutations in *MFN2* cause the peripheral neuropathy Charcot-Marie-Tooth type 2A (CMTIIA). It affects sensory and motor neurons of the distal extremities, resulting in the degeneration of long peripheral nerves (Zuchner et al., 2004). Clinical symptoms include distal weakness of the lower limps, sensory loss, decreased reflexes and foot deformities (Lawson et al., 2005). The disease causing mutations are predominantly missense mutations that cluster in the GTPase and RAS-binding domains of the protein (Kijima et al., 2005; Zuchner et al., 2004).

There are no inherited diseases that are directly linked to essential fission components so far. There has been a single case reported however of a newborn girl that carried a dominant-negative mutation in DRP1. The girl died 37 days after birth and exhibited, among other symptoms, severe neurological defects, including microencephaly, abnormal brain development and optic atrophy (Waterham et al., 2007).

Apart from diseases caused by perturbations of the mitochondrial fusion proteins, mitochondrial dynamics participate in a variety of other neurodegenerative diseases including Parkinsons's disease, Alzheimer's disease and Huntington's disease (Chen and Chan, 2009). Like ADOA and CMTIIA many of these pathologies affect distinct region of the brain and peripheral nervous system further emphasizing the importance of mitochondrial dynamics for neuronal health.

1.5.2. In Cancer

Mitochondria in the context of cancer have mainly received attention due to the Warburg effect, which describes the metabolic switch to glycolysis that tumor cells undergo as part of their escape mechanisms from apoptosis due to the hypoxic tumor environment. Beyond Warburg, there are multiple pathophysiological mechanisms how mitochondria may be involved in tumor development. Likewise, increasing evidence also supports a link between dysregulated mitochondrial dynamics and cancer development and progression.

Analyses of various cancer cells revealed up- or down-regulated levels of proteins mediating mitochondrial fission and fusion highlighting a role of unbalanced fission and fusion in tumorigenesis. DRP1 levels were found to be upregulated reflected by fragmented mitochondria in metastatic breast cancer cells (Zhao et al., 2013). Strikingly, silencing DRP1 suppressed the metastatic nature of the cancer cells. Similarly, DRP1 dependent fission was associated with enhanced cancer cell migration and invasion (Ferreira-da-Silva et al., 2015; Zhao et al., 2013). In line with a pro-tumorigenic potential of the fission protein DRP1, downregulation of fusion mediating proteins, mitofusins and OPA1 are associated with enhance tumor growth and migration (Garber et al., 2001; Nagaraj et al., 2012; Zhao et al., 2013; Zhao et al., 2012). Strikingly, increased OPA1 levels were found to correlate with tumor stage, recurrence incidences, and chemosensitivity generally leading to a poor prognosis (Fang et al., 2012). Moreover, conventional chemotherapeutics were suggested to exert their function partially through an OPA1-dependent function as low OPA1 levels facilitate cell

death (Kong et al., 2014; Zhao et al., 2012).

The mechanistic involvement of mitochondria-shaping proteins in tumorigenesis and tumor migration is not clear however and will require further investigations.

1.6. Apoptosis

In multicellular organisms apoptosis is essential during embryonic development but also later during adult life to maintain normal cellular homeostasis. Interference with the core apoptotic pathway manifests as severe diseases (Danial and Korsmeyer, 2004). Insufficient apoptosis may result in cancer or autoimmunity, while its acceleration is associated with degenerative disease. Once initiated, apoptosis follows an evolutionary conserved program that is characterized by morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000).

Depending on cell type and stimulus two apoptotic pathways can be identified: the intrinsic pathway which is initiated by internal events such as DNA damage and the extrinsic pathway that is triggered by an external signal acting at plasma membrane receptors for example involving the Fas ligand/Fas receptor (Scaffidi et al., 1999). Activation of these so called death receptors leads to initiator caspase-8 recruitment and activation which in turn propagates apoptosis by cleavage of the downstream effector caspases such as caspase-3 and -7 which propagate cell death (Walczak and Krammer, 2000).

Intrinsic and extrinsic death pathways may be intertwined at different levels. For example, caspase-8 that is activated upon death receptor triggering may result in cleavage in of the Bcl-2 family member Bid, which in turn translocates to mitochondria inducing a mitochondrial amplification loop of apoptosis. Moreover, both pathways eventually propagate apoptosis through triggering a cascade of proteolysis mediated by executioner caspases. This family of cysteine proteases exists as inactive proforms until activation. Apart from cleaving key substrates in the cell to produce many of the cellular and biochemical events of apoptosis, they cleave one another and amplify their activity (Degterev et al., 2003).

1.6.1. The Role of Mitochondria in Apoptosis

Intrinsic apoptosis almost always propagates through the involvement of mitochondria, which store numerous apoptogenic factors that, upon release induce caspase activation.

Members of the Bcl-2 protein family are critical regulators of apototic events occurring in mitochondria. It comprises pro- and anti-apoptotic members that share conserved α-helices in their structure (Danial and Korsmeyer, 2004). Proapoptotic members are further subdivided into multidomain and BH3-only proteins. The latter connect upstream proapototic signals to the mitochondrial pathway and once they are activated they serve as ligands for the multidomain proteins Bax and Bak (Eskes et al., 2000; Wei et al., 2000). Their subsequent translocation and homo/heterooligomerization on the OMM and coinciding cristae remodeling results in the release of apoptogenic factors from the mitochondria, most importantly cytochrome c (Frezza et al., 2006; Scorrano and Korsmeyer, 2003; Wei et al., 2000). The released cytochrome c, a key apoptotic protein, triggers the formation of the apoptosome complex consisting of cytochrome c/Apaf-1 and caspase-9 which subsequently propagates apoptosis by triggering effector caspases and cysteine proteases (Zou et al., 1999).

Mitochondria participate also actively in apoptosis as they function as crucial stress sensor of the cell. Depending on the stress level the cell endures mitochondria may be spared from autophagy and apoptosis by elongation or fragment promoting autophagy and more severely apoptosis (Gomes and Scorrano, 2008).

Coinciding with OMM permeabilization by Bax/Bak oligomerization mitochondrial function and morphology is altered. Functions such as the membrane potential that is required for OXPHOS and the calcium buffering capacity (Green and Kroemer, 2004) are affected, the network fragments (Frank et al., 2001a) and mitochondria undergo an ultrastructural rearrangement termed cristae remodeling (Suen et al., 2008; Wasilewski and Scorrano, 2009).

1.6.2. Cristae Remodeling and Cytochrome c Release

Cristae remodeling is a process describing the widening of cristae junctions and fusion of individual cristae (Frank et al., 2001a; Germain et al., 2005; Scorrano et al., 2002). Since apoptogenic factors are stored within the cristae, remodeling is a necessary process for their complete release into the cytosol. Due to its role in the apoptosome formation cytochrome *c* is a key protein in apoptosis as demonstrated by cells that lack cytochrome c or express a mutant form which fail to trigger the caspase cascade following OMM permeabilization (Hao et al., 2005). Cytochrome c is a water-soluble basic protein that is present as loosely and tightly bound pools attached to the inner membrane by its electrostatic interactions with IMM lipids. Oxidation in the course of apoptosis of the lipids releases cytochrome *c* but is insufficient for its complete release.

Cristae remodeling is induced by several proapoptotic BH3-only proteins including Bid, Bim-S, BNIP3, and Bik (Germain et al., 2005; Scorrano et al., 2002; Yamaguchi et al., 2008). The requirement of the BH3 domain is controversial as opposing results were shown (Scorrano et al., 2002; Yamaguchi et al., 2008). Moreover, although cristae remodeling requires BAX but not its activation, it seems to occur independently of OMM permeabilization (Yamaguchi et al., 2008). The exact molecular mechanism that triggers cristae remodeling is still unknown however. Oligomers of OPA1 that tighten cristae junctions are early targets during apoptosis resulting in their disruption, which coincides with the opening of the cristae junctions (Frezza et al., 2006). *In vitro* experiment with mutant forms of truncated Bid demonstrated that a α6 helix that inserts into the membrane, which is found in Bid as well as Nip3 and BimS are required for cristae remodeling (Cogliati et al., 2013). Thus, this helix may induce cristae remodeling by directly interacting with OPA1 oligomers, or since Bid was also shown to bind cardiolipin (Epand et al., 2002) it may act through membrane alterations. Considering that disassembly of OPA1 oligomers is a key event in cell death it represents a potential point for modulation of apoptosis.

1.6.3. Mitochondrial Morphology in Apoptosis

The permeabilization of the OMM coincides with fragmentation of the mitochondrial network (Frank et al., 2001a). Yet, their correlation is not clearly defined (Breckenridge et al., 2008; Ishihara et al., 2009. In fact, evidence suggests rather that they are distinct events that share common proteins {Sheridan, 2008 #4242; Parone et al., 2006; Sheridan et al., 2008). The fragmentation is mediated by both, inhibition of fusion and activation of fission in a DRP1-dependent manner (Lee et al., 2004).

DRP1 is recruited from the cytosol early during apoptosis and promotes the translocation and oligomerization of Bax promoting OMM permeabilization (Montessuit et al., 2010). Subsequent SUMOylation, which is increased during apoptosis, stabilizes DRP1 on the OMM (Wasiak et al., 2007). Equally, increased DRP1 translocation is associated with an increased susceptibility to apoptosis and alterations in the cristae structures (Costa et al., 2010). Moreover, expression of a dominant negative form of DRP1 (DRP1[K38A]) or chemical inhibition of DRP1 blocks fragmentation and slows down apoptosis (Cassidy-Stone et al., 2008; Frank et al., 2001a). DRP1 recruitment to mitochondria likely at least partially depends on hFis1 (James et al., 2003), which also participates in ER-mediated apoptosis (Alirol et al., 2006). Interestingly, silencing of DRP1 diminishes the release of cytochrome c linking OMM permeabilization with cristae remodeling (Estaquier and Arnoult, 2007).

Simultaneous with the Drp-1 mediated fission, apoptosis is amplified by suppression of MFN1 dependent fusion (Karbowski et al., 2004) and silencing of either mitofusin renders cells more susceptible to apoptosis while their overexpression can protect from apoptosis (Sugioka et al., 2004). Fusion inhibition is likely mediated by the pro-apoptotic Bcl-2 family members occurring before or simultaneously with the activation of Bax. Indeed, Bax and Bak are associated with MFN2 in healthy conditions and promote its activity (Karbowski et al., 2006). During apoptosis, the colocalization of MFN2 with Bax (Karbowski et al., 2002) is required for Bax-mediated OMM permeabilization (Scorrano, 2013) while Bak

dissociates from MFN2 and associates with MFN1 (Brooks et al., 2007). Recently published data suggests that the association of Bak with MFN1 is dependent on the phosphorylation status of MFN1. ERK-mediated phosphorylation of MFN1 at a site required for its fusogenic function stimulates the binding to Bak, prevents mitochondrial fusion and renders cells more susceptible to apoptosis (Pyakurel et al., 2015).

1.6.4. Inducing Cell Death by Targeting Intrinsic Apoptosis

Apoptosis is a natural barrier to proliferation and thus a crucial anticancer defense. The dysregulation in the involved mechanisms represents one of the major hallmarks in cancer development as it allows uncontrolled cell growth. Currently, the intrinsic apoptotic program is more widely implicated as barrier to cancer pathogenesis (Hanahan and Weinberg, 2011) yet also as one of the most deregulated pathways (J Lopez, 2015). Tumor cells evolved numerous strategies to limit or overcome apoptosis which include loss of tumor suppressors, activation of proto-oncogenes, upregulation of antiapoptotic regulators such as Bcl-2 and downregulation of proapoptotic facors like Bax and Bim (Hanahan and Weinberg, 2011).

Many conventional anti-cancer therapies like etoposide, cisplatin or doxorubicin induce apoptosis, albeit in indirect ways. Cancer cells often become resistant to these treatments through modifications of the apoptotic mechanisms (Fulda and Debatin, 2006). Hence, drugs that specifically and directly target mitochondria and downstream pathways are becoming of great interest. Examples for these are drugs that directly induce a loss of mitochondrial membrane potential, like betulinic acid (Debatin et al., 2002) or SMAC-mimetics that are designed to enhance caspase activity (Li, 2004).

Examples of mitochondria targeted drugs include aurilide, which targets the protease complex that prohibitin 1 forms with the ATP-dependent protease spastic paraplegia 7 (SPG7) (Sato et al., 2011). The resulting increase in processing of the proapototic OPA1

facilitates apoptosis in treated cells. Interestingly, aurilide has already been reported to be cytotoxic for cancer cells (Han et al., 2006).

Also the mitochondrial division inhibitor-1 (mdivi-1) targets mitochondrial dynamics as it selectively inhibits DRP1 (Cassidy-Stone et al., 2008). It attenuates DRP1 self-assembly and results in inhibition of mitochondrial fission. Indeed mdivi-1 was found to have *in vivo* efficacy due to cell arrest against lung tumors that have increased DRP1 levels (Rehman et al., 2012). There is increasing evidence however that mdivi-1 may have many off-targets, such as preventing mitochondrial outer membrane permeabilization and effects on ion currents and cell membrane potential possibly accounting for many effects observed upon treatment (Kushnareva et al., 2012; Qian et al., 2013).

In this context cristae remodeling may represent a promising target to facilitate cytochrome *c* release from the mitochondria to induce the downstream caspase cascade (Cereghetti and Scorrano, 2006). The identification of pharmacological agents to treat the antiapoptotic proteins OPA1 ought to help in the validation of this approach.

1.7 OPA1 - a Multifunction Dynamin-like GTPase

1.7.1. The Family of Dynamin-like GTPases

In the cell, membrane fusion and fission are constant ongoing mechanisms to rearrange the complex structure of organelles and the cell as a whole during different processes, such as cell division, endo- and exocytosis, organelle restructuring. Large GTPases belonging to the dynamin superfamily oftentimes mediate these processes by catalytically promoting the division or fusion of cellular membranes. This is achieved by catalyzing a thermodynamically unfavorable reaction using the energy that is derived from the hydrolysis of GTP. Their

conservation throughout animals, plants and prokaryotes (van der Bliek, 1999) demonstrates their importance for physiology. The name-giving as well as the most studied protein of this family, dynamin-1 (van der Bliek and Meyerowitz, 1991), mediates scission of clathrin-coated endocytic vesicles but also participate at several other fission sites including budding of caveolae, phagocytosis and during actin rearrangements (Sever et al., 2013). Other members of the family, the dynamin-like proteins (DLPs), share common features with classical dynamins that set them apart from other GTPases such as the Ras or the heterotrimeric Gα families (Song and Schmid, 2003). Moreover, all members act as mechano-enzymes that mediate intracellular membrane remodeling through GTP hydrolysis. Apart from the highly conserved GTPase domain all of them contain a middle domain consisting of two helical regions and the GTPase effector domain (GED) that together form the stalk (Ford et al., 2011; Low et al., 2009) and a highly variable region termed insert B (van der Bliek, 1999). These domains cooperate *via* both intra- and intermolecular interactions to promote the self-assembly of higher order structures.

The globular GTPase domain consists of a mixed six-stranded beta-sheet that is surrounded by five alpha-helices (Leipe et al., 2002) and includes the typical GTP binding motifs (G1-G4) needed for phosphate binding (P-loop: GXXXXGKS/T), coordination of Mg^{2+} (DXXG) and nucleotide binding (T/NKXD) (Dever et al., 1987). Interestingly, large GTPases harbor a GTPase domain that contains almost twice the number of amino acids (aa) of about 300 aa compared to the minimal GTPase domain of Ras of about 160 aa (Niemann et al., 2001). Their GTPase activity is usually stimulated by self-oligomerization into dimers or tetramers that subsequently form higher order oligomers to form rings or helices (Bramkamp, 2012). The middle domain and GED cooperate to form a helical bundle, called the stalk (Faelber et al., 2011; Ford et al., 2011; Low et al., 2009), which mediates oligomerization and regulates GTPase activity in all described cases. It mediates the formation of a dimer, which then form into a dimer dimer formation (Fröhlich et al., 2013). Dimers and higher order oligomers are stabilized by GED/GED and GED/GTPase domain interactions (Smirnova et al., 1998). Also OPA1 and Mgm1 were shown to oligomerize,

yet without detailed structural analysis. They are thought not to be obligate dimers but to assembly similarly via their stalks into higher order fusion promoting structures (Ban et al., 2010; DeVay et al., 2009).

The insert B region is the most variable component of DLPs and is situated at the end of the stalk. In dynamin this region forms a pleckstrin homology domain, which mediates binding to membranes (Faelber et al., 2011; Ford et al., 2011). Similarly, the insert B regions in OPA1 may bind cardiolipin (Ban et al., 2010; DeVay et al., 2009). In mitofusins this region consists of a set of transmembrane helices that anchor these proteins into the OMM (Tyler J Moss, 2011). In Drp this region was proposed to mediate interactions with the OMM protein Mff (Liu and Frazier, 2015; Strack and Cribbs, 2012).

In dynamin the middle and GE domain contain a bundle signaling element (BSE), a linker region that mediates conformational change (Chappie et al., 2010). It is a three helix bundle structural element that regulates a dimer interface between two GTPase domains of two proteins (Chappie et al., 2011). The GTPase domain interface formation is GTP dependent and required for oligomerization dependent GTP hydrolysis (Chappie et al., 2011). This BSE is thought to be an essential structural element in the conformational change required for the membrane remodeling (Low et al., 2009). BDLP and MxA also harbor a BSE, but most DLPs like atlastin, mitofusins and OPA1 have no defined BSE (Bramkamp, 2012). The coiled coil domain found in OPA1 between the TM domain and the GTPase domain might however serve as BSE (Bramkamp, 2012).

Generally, DLPs have a low affinity for GTP as well as GDP compared to Ras or Gα proteins (Eccleston et al., 2002). The dissociation rate of GDP from dynamin is fast in comparison GDP release, making it unlikely to be the rate-limiting step in the dynamin GTPase cycle. Importantly, the intrinsically fast GDP release renders a nucleotide exchange factor (GED) *in vivo* unnecessary (Hanna Damke, 2001) and they are believed to be constitutively loaded with GTP in physiological conditions (Song and Schmid, 2003). This may however depend on the availability of local nucleotide pools that are stabilized by nucleoside diphosphate kinases (NDPKs) that produce GTP through ATP driven conversion of GDP (Boissan et

al., 2014).

The stimulation of hydrolysis by oligomerization (Hanna Damke, 2001) is complemented by stimulation through membrane recruitment. Many members of the family also harbor a pleckstrin-homology (PH) domain (excluding mitofusins, MxA, GBP, atlastin, and OPA1 which mediates interaction with negatively charged lipid membraned (Bramkamp, 2012) serving as targeting domain. Moreover, classical dynamins carry a C-terminal proline-rich domain (PRDs) that binds to SRC-Homology-3 (SH3) domains to mediate protein-protein interaction with possible modulators of dynamin (Sundborger et al., 2014). Interestingly, only atlastin, mitofusins and OPA1 contain a transmembrane domain that anchors them into their respective membrane. As a result they obviously lack the versatile involvement in different processes like other DLPs and may hence do not rely on membrane recruitment for activation but rather may continuously mediate membrane remodeling.

1.7.2. The Power Stroke - Mechanisms of Membrane Fission and Fusion

The mechanism underlying fission has been well defined mainly through work done on dynamin. To induce membrane fission, it forms helices around the division site by self-oligomerization and through a GTP dependent conformational change leading to a rearrangement of the formed rings it constricts and fission occurs (Ford et al., 2011).

While the mechanism of DLP-mediated fission is becoming clear, the mechanism of fusion is remains uncertain. Our current knowledge indicates that membrane fusion is not mediated through helical structures like fission but rather through trans-interactions between the GTPase domains of proteins anchored in opposing membranes. In line with this, both atlastin and mitofusins were reported to be required on both membranes for fusion to occur (Meeusen et al., 2004). Structural studies of atlastin revealed two dimeric conformations that may represent a pre- and a post-fusion state (Byrnes et al., 2013). According to this model, GTP binding tethers the two opposing membranes through G domain dimerization while subsequent hydrolysis induces membrane fusion through an

intramolecular conformational change (Byrnes et al., 2013). This results in a decreased distance between the two membranes which is critical as longer linkers impair fusion (Pendin et al., 2011) Once in close proximity, membrane curvature and stress mediated by the transmembrane domains of atlastin facilitate membrane fusion (Tina Y Liu, 2012; Tyler J Moss, 2011). Whether membrane fusion occurs spontaneously or upon release of the tether remains to be demonstrated. The proposed fusion mechanism interestingly resembles very much that of fission by enforcing membrane curvature through constriction or tubulation (Byrnes et al., 2013). Although some exact details of the mechanism are still unknown, the elucidated principles may be indicative for other fusion mechanisms involving mitofusins and OPA1. Interestingly however, OPA1 is apparently not required on both opposing membranes to mediate fusion (Song et al., 2009) pointing out a crucial difference to atlastin, whereas that its yeast homologue Mgm1 that is thought to function like OPA1 is required on both membranes (Meeusen et al., 2006). Future structural studies on OPA1 will elucidate how it fuses membranes.

1.7.3. OPA1

In 1992 the yeast homolog of OPA1, Mgm1 was identified as GTP binding protein that is required for mtDNA maintenance in *S. cerevisae* (Jones and Fangman, 1992) and later with the same function in budding yeast (*S. pombe*), Msp1 (Pelloquin et al., 1998). Mutations in the human homologue *OPA1* are associated with autosomal dominant optic atrophy (ADOA) (Alexander et al., 2000; Delettre et al., 2000). Like its yeast homologues, also OPA1 is required for mtDNA maintenance but also participates in additional mitochondrial functions. It mediates the fusion of the IMM (Cipolat et al., 2004; Olichon et al., 2003; Song et al., 2009) and is a key mediator of cristae morphology controlling respiration (Cogliati et al., 2013) and cytochrome *c* release (Frezza et al., 2006).

Gene and Protein

The human *OPA1* gene is located on chromosome 3q28-29 and spans about 100 kilobases containing 30 coding exons including three alternative exons (4, 4b, 5b). Alternative splicing of the mRNA creates 8 isoforms (Delettre et al., 2001) whose individual functions are still unclear. There are organ-specific variations in expression of the different splicing variants found suggesting a regulatory mechanism through alternative splicing (Delettre et al., 2001; Olichon et al., 2007a). The most predominant and thus most studied are isoform 1 and isoform 7 (Olichon et al., 2007a). OPA1 transcripts are expressed ubiquitously in all tissues, though they are most abundant in retina, brain, testis, heart and muscle (Alexander et al., 2000). Interestingly, among the alternatively spliced exons only exon 4 is retrieved at least partially in yeast and invertebrates, whereasexons 4b and 5b are only found in vertebrate genomes and are absent in lower eukaryote genomic sequences (Olichon et al., 2007a). They appear to be crucial for OPA1 function as ADOA mutation spare exons 4b and 5b.

Exon 4b containing mRNAs (present in isoforms 3, 5, 6, and 8) are highly expressed in the brain, while exon 5b containing mRNAs (present in isoforms 5, 6, 7, and 8) are largely present in liver, kidney, and thymus suggesting a tissue-specific role of the different isoforms. Interestingly, exon 4b and 5b were hypothesized to specifically participate in trapping cytochrome *c* inside the mitochondria. They may have resulted from co-evolution into a vertebrate-specific mechanism to control cytochrome c segregation (Olichon et al., 2007a). This may also provide an explanation for why OPA1 adopted the role in apoptosis that Mgm1 or Msp1 do not have (Olichon et al., 2007a). Moreover, in line with the role of OPA1 in apoptosis through oligomerization (Frezza et al., 2006) domain 5b encodes for a predicted coiled-coil domain that may mediate protein-protein interactions (Olichon et al., 2007a).

Upon import into the mitochondria mediated by an N-terminal mitochondrial import signal the long membrane anchored (l-OPA1) forms are partially processed to yield shorter

forms of OPA1 (s-OPA1) by proteolytic cleavage (Ishihara et al., 2006).

An N-terminal transmembrane domain mediates Anchorage of the long forms into the IMM with the protein facing towards the intermembrane space (Delettre et al., 2000; Olichon et al., 2002). The following protein domains vary among isoforms due to alternative splicing (Olichon et al., 2007a). Domains encoded by the constitutive exon 5 and the alternative 5b contain cleavage sites S1 and S2 for the proteases that mediate OPA1 processing (Ishihara et al., 2006). Hence, isoforms may be processed once or twice depending on which exons they encode. A highly conserved GTPase domain follows this variable region and the dynamin like GTPase specific stalk consisting of middle domain, insert B and a GE domain (Belenguer and Pellegrini, 2013). Also in OPA1, like in other dynamin like proteins the stalk is thought to function in homo-oligomerization and stimulation of GTPase activity.

Regulation of OPA1

Both OPA1 and its yeast homologue Mgm1 are regulated by proteolytic mechanisms. Proteolytic processing of their long forms that are anchored into the IMM through an N-terminal TM domain results in a shorter soluble form without membrane anchoring function (Esser et al., 2002; Herlan et al., 2003; Ishihara et al., 2006). Constitutive cleavage of OPA1 keeps the balance between long and short forms steady in normal conditions. Changes in the equilibrium or stress induced cleavage results in drastic changes of mitochondrial structure (Griparic et al., 2007; Ishihara et al., 2006; Song et al., 2007). OPA1 carries two identified cleavage site, S1 and S2 of which S1 is found in all isoforms while S2 is only present in isoforms carrying exon 5b (i.e. in only 4 of the eight forms, isoform 7 being one of them) (Ishihara et al., 2006; Satoh et al., 2003). The cleavage sites are recognized by the IMM peptidase Oma1 and the IMS i-AAA protease Yme1L (Ehses et al., 2009; Head et al., 2009; Song et al., 2007). While the latter mediates constitutive processing at the proteolytic site S2 (Song et al., 2007; Stiburek et al., 2012) presumably during OPA1 import and sorting (Griparic et al., 2007), the former processes OPA1 at S1 in events of stress such

as mitochondrial depolarization or reduction in ATP levels (Duvezin-Caubet et al., 2006; Guillery et al., 2008).

Loss of the constitutive processing of OPA1 by genetic ablation of Yme1l causes fragmentation, disordered cristae morphology and susceptibility to apoptosis (Stiburek et al., 2012). Thus the short form and/or the balance of short and long forms is crucial for OPA1-mediated functions in the cells. Moreover, loss of OPA1 forms that contain exon 5b (containing the site for Yme1L cleavage) show a mitochondrial morphology that resembles that of pre-apoptotic cells without an impaired fusion however (Griparic et al., 2007). This may support the aforementioned proposed role of exon 5b containing isoforms in apoptosis (Olichon et al., 2007a).

Stress-induced processing of OPA1 by Oma1 appears to be a mechanism that participates in the overall mitochondrial quality control system. An increase in Oma1-processed OPA1 short forms leads to mitochondrial fragmentation and clearance of damaged organelles by mitophagy or apoptosis (Baker et al., 2014; Quirós et al., 2012). Interestingly, loss of Oma1 does not impair mitochondrial morphology (Ehses et al., 2009; Head et al., 2009) suggesting that the Oma1 processed form of OPA1 is not required for fusion and that it might even participate in fission itself (Anand et al., 2014). The physiological importance of stress induced Oma1 cleavage is highlighted by a mouse model deficient in Oma1, which displays a strong metabolic phenotype perhaps related to the role of OPA1 has in energy production (Quirós et al., 2012).

Apart from the aforementioned proteases also Parl, an inner membrane rhomboid protease participates in OPA1 processing (Cipolat et al., 2006). Cells of Parl knockout mice are more susceptible to intrinsic apoptosis, which can be compensated by expressing a soluble form of OPA1. As Parl knockdown does not affect OPA1 maturation or proteolysis per se (Griparic et al., 2007), an indirect role in OPA1 processing appears likely (Ishihara et al., 2006), Parl being responsible for the further processing of short OPA1 to generate the soluble forms. A recent study proposed a link between MAMs and OPA1 processing:

additional cleavage products of OPA1 were found in starving liver, where mitochondria and ER were more associated in a MFN2-dependent fashion (Aditi Sood, 2014). The relevance of these cleavage forms and the involved control mechanisms are still to be elucidated however.

Little is known on OPA1 oligomerization and distribution within the microdomains of mitochondria. Yet, the distribution of long and short forms of its homolog Mgm1 differs. While the long form is predominantly retrieved at the cristae membrane, the short form is found also in the IMS and in proximity to the OMM (Zick et al., 2009a). Yet, both OPA1 and Mgm1 short and long and were shown to form homo- and hetero-oligomers (Zick et al., 2009a). Interestingly, *in vitro* studies showed that only oligomers of Mgm1 formed in trans by long and short forms display GTPase activity (DeVay et al., 2009) and that OPA1 GTPase activity is stimulated in vitro in conditions facilitating random aggregation to simulate oligomerization by low salt concentrations (Ban et al., 2010). Both long and short forms of OPA1 form high molecular weight complexes that participate in multimolecular complexes important for cristae structure (Frezza et al., 2006; Yamaguchi et al., 2008). They are crucial in regulating cristae shape in healthy cells and are early targets during apoptosis when their disruption coincides with cristae remodeling (Frezza et al., 2006). Oligomer disruption by stress conditions including apoptotic stimuli leads to conversion of l-OPA1 into s-OPA1 inducing mitochondrial fragmentation (Baricault et al., 2007; Duvezin-Caubet et al., 2006; Guillery et al., 2008). Conversely, stabilized OPA1 complexes mediate cristae tightening, promoting efficient respiration by favoring respiratory chain supercomplex stability (Cogliati et al., 2013) and enhancing ATP synthase assembly (Patten et al., 2014). Considering that OPA1 oligomers are in a large complex with other proteins, OPA1 activity and oligomerization may also depend on other members of these complexes as it was shown for SLC25A (Patten et al., 2014). In conclusion, oligomerization adds an important regulatory aspect to OPA1 biology, as it appears critical for its functions as an antiapoptotic protein and in regulating efficient ATP production. Indeed, both of these functions are distinct from the fusogenic nature of OPA1 (Cogliati et al., 2013; Frezza et al., 2006).

Whether OPA1 is subjected to posttranslational modifications like Mitofusin and DRP1 is still unclear. Deacetylation of two lysine residues in the GE domain of OPA1 by Sirt3 was suggested to activate OPA1 to mediated fusion and its antiapoptotic function (Samant et al., 2014). The same regulation seems to be exerted upon OPA1 phosphorylation by protein kinase C (PKC) (Fang et al., 2012). However, the exact mechanism and relevance of these modifications remain to be investigated.

Functions

Mice that constitutively lack *OPA1* die early during embryogenesis, around E8.5, demonstrating the physiological important role of OPA1 in development (Alavi et al., 2006; Davies et al., 2007).

Apart from the aforementioned role in fusion, genetic *OPA1* ablation severely alters mitochondrial ultrastructure. This consequently sensitizes cells to apoptotic stimuli and reduces respiratory chain efficiency eventually leading to cell death and impairment of cell growth. Conversely OPA1 overexpression protects from tissue damage by counteracting cell death and boosting OXPHOS efficiency, as demonstrated by a mouse model with controlled OPA1 overexpression (Varanita et al., 2015).

OPA1 in Mitochondrial Fusion

As aforementioned, OPA1 promotes mitochondrial fusion together with the OMM GTPase MFN1(Cipolat et al., 2004). The fusogenic function of OPA1 relies on its GTPase activity as mutating amino acids in conserved regions of the GTPase domain result in fragmented mitochondria (Cipolat et al., 2004; Frezza et al., 2006). Similarly, ablation of OPA1 results in increased mitochondrial fission (Cipolat et al., 2004).

Whether both long and short forms of OPA1 are required for mitochondrial fusion is still debated. It has been suggested that both forms are needed for successful fusion to occur

(Song et al., 2007) with long OPA1 functioning as tether in trans and short OPA1 performing the fusogneic activity, as it is the case for Mgm1 (DeVay et al., 2009; Herlan et al., 2003; Zick et al., 2009a). Whether the function of Mgm1 reflects what is happening in mammalian cells however is doubtful. Particularly, considering different from in mammalian cells, in yeast cells OMM and IMM are not separable events and requires an additional protein, Ugo1p for fusion to occur (Anton et al., 2011). Furthermore, data obtained on OPA1 challenges the concept obtained from Mgm1 showing that only the long form of OPA1 is fusogenic and sufficient to promote mitochondrial fusion (Ishihara et al., 2006; Song et al., 2009). Also noncleavable long OPA1 is sufficient to promote stress induced mitochondrial hyperfusion in OPA1$^{-/-}$ cells (Tondera et al., 2009). In line with this the genetic ablation of both proteases that process OPA1, Yme1l and Oma1 does not prevent mitochondrial fusion further suggesting the long form of OPA1 is sufficient to promote fusion (Anand et al., 2014b). The short form of OPA1 instead was proposed to possibly interact with MFN1 to coordinate fusion rather than promoting it or to maintain the physical link between the OMM and IMM since it was found to localize to the OMM on the IMS side (Cipolat et al., 2004; Satoh et al., 2003). Also *in vitro* a recombinant form of s-OPA1 was only able to tubulate liposomes yet not to fuse them supporting such model (Ban et al., 2010). Due to the coolocalization of short OPA1 with mitochondrial fission sites a possible role in fission is also debated (Anand et al., 2014). How this incorporate with the debated role of OPA1 in fusion remains to be elucidated. Interestingly, and despite its clearly defined role in fusion overexpression of OPA1 was oftentimes reported to lead to a dominant negative effect resulting in mitochondrial fragmentation instead of the expected hyperfusion (Cipolat et al., 2004; Olichon et al., 2007a). This appears to be a level-dependent effect however since, while high overexpression is associated with fragmentation, a mild overexpression slightly increases mitochondrial length (Cipolat et al., 2004).

OPA1 in Mitochondrial Ultrastructure

Striking evidence supports a key role of OPA1 in the control of cristae shape during physiology but also during apoptotic cristae remodeling as discussed in more detail below.

In cells devoid of OPA1 cristae are unorganized and their width is increased (Frezza et al., 2006; Griparic et al., 2004; Olichon et al., 2003) similar to mitochondria in the optic nerve of mouse models for ADOA (Alavi et al., 2006). Conversely, overexpression of OPA1 increases cristae number and reduces cristae width (Cogliati et al., 2013). OPA1 seems to tighten cristae through its membrane anchored form without necessity of the short form as in mice lacking the two OPA1 processing proteases Yme1L and OMA1 in which short forms of OPA1 are reduced, cristae appear unaltered (Anand et al., 2014). This is further supported by the fact that that l-OPA1 and not s-OPA1 can compensate for prohibitin which depletion renders cells more susceptible to apoptosis due to a loss of l-OPA1 forms (Merkwirth et al., 2008). While it was suggested that OPA1 might sit at the cristae junctions as their width corresponds to that of rings formed by the homologue dynamin I (Frezza et al., 2006) EM analyses suggested that it may be distributed throughout the cristae (Zick et al., 2009a)(Zick 2009). This would rather suggest a distribution of OPA1 oligomers along the length of the cristae. Apart form OPA1 also other proteins have been implicated to participate in the shaping of IMM cristae and their remodeling such as ATP synthase dimerization (Cogliati et al., 2013; Zick et al., 2009a) yet OPA1 appears to be a key determinant in cristae structure.

OPA1 in Mitochondrial Respiration

Numerous studies link OPA1 with OXPHOS efficiency relating it to the roles of OPA1 in mitochondrial ultrastructre and fusion. In particular, a variety of ADOA cases were found to display similarities with other mitochondrial encephalomyophathies with a decrease in ATP production and complex I fed respiration (Amati-Bonneau et al., 2005; Amati-Bonneau et al., 2008; Lodi et al., 2004; Zanna et al., 2008).

Respiratory chain complexes localize to in the IMM inside the cristae (Vogel et al., 2006) where they assemble into supercomplexes for efficient ATP production (Acin-Perez et al., 2008; Lapuente-Brun et al., 2013). These are dynamic complexes composed of different ratios of the complexes of the respiratory chain. In line with the role OPA1 has in maintaining cristae ultrastructure it also plays a role in their assembly into respiratory chain supercomplexes (Cogliati et al., 2013). In fact, the width of cristae directly reflects on their assembly - as cristae tighten supercomplexes are favored and conversely a decrease in cristae width reduces supercomlexes. Inefficient assembly of supercomplexes results in less efficient ATP production driven by complex I substrates (glutamate/malate) as it was also observed to be the case in fibroblasts isolated from ADOA patients (Zanna et al., 2008). Moreover, also ATP synthase assembly appears to increase in tighter cristae ensuring efficient respiration (Patten et al., 2014).

Like its homologues, also OPA1 was also suggested to play a role in mtDNA maintenance. A resulting defect in the biosynthesis of the mtDNA-encoded complexes of the respiratory chain complexes may thus contribute to a respiratory defect. Chronic reduction of OPA1 by genetic ablation or by mutations causing truncated proteins results in a reduction in mtDNA levels which is suggested to at least partially account for defects in cellular respiration (Chen et al., 2010; Cogliati et al., 2013). Ther a mtDNA defect is likely to originate from a fusion defect as it was also reported to occur in MFN1^{-/-}, MFN2^{-/-} cells (Chen et al., 2010). Interestingly mtDNA integrity in ADOA patients was reported to be affected particularly in cases of OPA1 missense mutations resulting in an accumulation of deletions (Amati-Bonneau et al., 2008). How defective OPA1 may account for mtDNA deletions and whether it contributes or is rather a result of the OXPHOS defect remains to be elucidated.

OPA1 in Cristae Remodeling and Apoptosis

During apoptosis the OMM is permeabilized under the control of Bcl-2 family members: OMM permeabilization is accompanied by organelle fragmentation and cristae remodeling (Danial and Korsmeyer, 2004; Wasilewski and Scorrano, 2009), for example induced by pro-apoptotic BH3-only BCL-2 family members such as Bid, Bim-S and Bnip3 (Landes et al., 2010; Scorrano et al., 2002; Yamaguchi et al., 2008).

OPA1 high molecular weight oligomers are early targets of BID, BIM-1 and BNIP3 as well as of intrinsic death stimuli (Frezza et al., 2006) by which they are disrupted and cleaved. The structural integration of Bnip3 into the OMM with an extrusion of a small amino acid stretch into the IMS suggest that a physical interaction may be possible which is strengthened by *in vitro* experiments showing a BNIP3 interaction with OPA1 (Landes et al., 2010). OPA1 oligomer disruption coincides with cristae remodeling and results in a redistribution of apoptotic factors as well as the disassembly of respiratory supercomplexes resulting in impaired mitochondrial function. Not surprisingly, OPA1 ablation increases the apoptotic sensitivity of cells (Cipolat et al., 2006; Olichon et al., 2003). Conversely, a mild overexpression of OPA1 confers resistance to apoptosis (Frezza et al., 2006; Varanita et al., 2015). Supporting a role in cristae remodeling, cells isolated from ADOA patients are more sensitive to apoptosis (Formichi et al., 2015; Olichon et al., 2007b). Moreover, recent results support the role of OPA1-regulated cristae remodeling in cell sensitivity to apoptosis as a mild increase in OPA1 level protects mice from apoptosis-inducing tissue damage *in vivo* (Costa et al., 2010; Varanita et al., 2015).

Whether both, the long and the short form (Merkwirth et al., 2008), or selectively the short (Cipolat et al., 2006) or the long form (Anand et al., 2014) confer resistance to apoptosis is still under debate however. Genetic depletion of both proteases, Yme1L and OMA1 confer apoptotic resistance to cells (Anand et al., 2014) suggesting that the long forms of OPA1 are responsible for the antiapoptotic function of OPA1. Similarly, lack of OMA1 protects from

apoptosis, however only marginally (Anand et al., 2014; Quirós et al., 2012) implying that Yme1L-cleavage may be importenat. However, Yme1L kockout cells have a severely increased susceptibility to apoptosis arguing for the importance of both, long and short forms of OPA1 (Anand et al., 2014; Stiburek et al., 2012). Future experiments will bring clarity to the roles of OPA1 forms in apoptosis. One may speculate that the steady state balance of long and short forms of OPA1 mediated by Yme1L is required for the antiapoptotic role, while stress-induced processing through OMA1 ablates the protective function of OPA1. Whether the stress induced short form of OPA1 however has a stimulatory role in apoptosis, as it was suggested (Anand et al., 2014) or the protection simply relies on stabilization of the long form remains to be verified.

Despite the beneficial role of OPA1 up regulation there appears to be a level dependency since high levels are toxic (Cipolat et al., 2004) and strain-specific increases in the prevalence of cancer in OPA1 overexpressing mice (Varanita et al., 2015) indicate a disadvantageous aspect of increased OPA1 levels.

OPA1 in Cancer

Mitochondrial dynamics is receiving more attention also in the context of cancer development and progression.

Higher protein levels of OPA1 have been reported in lung adenocarcinoma cells (Fang et al., 2012; Garber et al., 2001). Strikingly, higher OPA1 levels correlated with an increased resistance to conventional chemotherapeutics such as cisplatin and a lower survival rate (Fang et al., 2012; Kong et al., 2014; Zhao et al., 2012). Interestingly, in a *Drosophila melanogaster* model for tissue overgrowth induced by hyperactivation of the Hippo pathway, that regulates cell size and tissue growth, OPA1 was found to be upregulated coinciding with elongated mitochondria. Attenuation of mitochondrial fusion by ablating OPA1 could rescue the tissue overgrowth suggesting that OPA1 participates observed phenotype (Nagaraj et al., 2012).

Moreover, considering the important role of OPA1 in regulating cytochrome c release it may also be an interesting target for future therapies to induce apoptosis. Indeed, knockdown of OPA1 in hepatocellular carcinoma cells sensitized them to cytotoxic treatment (Zhao et al., 2012) and indirect Akt-dependent downregulation of OPA1 facilitated cytochrome *c* release and apoptosis in lung cancer cells (Cho, 2011).

Pathological OPA1 Mutations – ADOA

Autosomal dominant optic atrophy, also known as Kjer's optic atrophy type 1, is the one of the most prevalent form of inherited optic atrophy (Kjer et al., 1996). Clinically it is characterized by a decrease in sight, tritanopia, central visual field defects and optic nerve pallor. Penetrance and clinical history are highly variable, suggesting that epigenetic or environmental factors contribute to the pathogenesis of the disease. ADOA plus forms exist, being clinically evident as a multi-systemic disorder associated with OPA1 mutations and resulting mtDNA instability (Amati-Bonneau et al., 2008). Patients suffering from ADOA plus show additional moderate deafness, ptosis and ophthalmoplegia.

The diversity in symptoms is also reflected by the considerable diversity found in OPA1 mutations. The mutations causing ADOA were firstly mapped to the gene encoding for OPA1 in 2000 by two groups who identified the first 8 mutations (Alexander et al., 2000; Delettre et al., 2000). In the meantime the spectrum of mutations in OPA1 resulting in ADOA has been broadened and shows an uneven distribution throughout the protein. Mutations range from premature truncating ones (40%), splice variant affecting ones (27%), missense mutations (27%), deletions (5%) and duplications (1%) (Amati-Bonneau et al., 2009). Thus, there is a considerable preponderance of nonsense mutations leading to truncated OPA1 polypeptides. In depth analysis of the distribution of the mutations showed a clustering within the higly conserved GTPase domain and the C-terminal region (Ferre et al., 2005; Pesch et al., 2001) emphazising the importance of these domains for OPA1 function. Up until today however no clear genotype/phenotype relationship has been possible to draw

since neither the type of mutation nor the location of the mutation seem to follow conclusive patterns (Pesch et al., 2001).

Physiopathology of ADOA

In line with the aforementioned diversity in mutations and clinical picture, the mechanism that underlies ADOA and how it relates to mutations in OPA1 still remains unclear. Haploinsufficiency was thought to explain the ADOA phenotype (Alexander et al., 2000). This appears conclusive considering the preponderance of many mutations leading to premature translation terminations that are assumed to be unstable and to be degraded quickly (Alavi et al., 2006). This received further support by the lower levels of OPA1 disease transcripts in ADOA patients compared to the ones of the healthy allele (Pesch et al., 2001). The decrease in OPA1 protein levels might thus significantly affect cells that usually have a comparable high OPA1 level. While the haplosufficiency appears to hold true for the mutations the cause premature stop codons, the possibility of a dominant negative effect has been proposed with regard to the missense mutations. Interestingly, missense mutations in OPA1 are associated mostly with the multisystemic forms of the disease (Amati-Bonneau et al., 2009).

OPA1 has multiple functions, thus one can speculate about many possible causes for the pathologic mechanism. Mitochondrial fusion and ultrastructure are closely related, with OPA1 being important for both. Thus, one would expect to observe strongly fragmented mitochondria in cells with impaired or mutated OPA1. This however was only the case in some of the cells that have been isolated and assessed from patients (Amati-Bonneau et al., 2005; Amati-Bonneau et al., 2008; Zanna et al., 2008). Nevertheless a correlation between the severity of the disease and increased fragmentation has been proposed and cells isolated from mouse models for OPA1 mutant forms that develop an ADOA like phenotype also show more fragmented mitochondria (Alavi et al., 2007; Davies et al., 2007). In agreement

with the role of OPA1 in stabilizing respiratory chain supercomplexes, mutations inducing haploinsufficiency do not influence the mitochondrial network *per se* but impair it when grown in galactose medium which forces mitochondria dependent ATP production (Olichon et al., 2006; Zanna et al., 2008). Fibroblasts isolated from ADOA patients behave the same, with fusion inhibition and an aberrant ultrastructure as well as impaired OXPHOS and ATP production (Lodi et al., 2004). Hence, a decrease in OPHOS capacity below a certain threshold that is critical for the functional integrity of RGC may account for the pathomechanism (Pesch et al., 2001). Apart from its role in stabilizing respiratory chain supercomplexes, OPA1 is important for stability of mtDNA, and ADOA mutations were associated with mtDNA instability (Hudson et al., 2007). In line with this hypothesis ADOA shows several similarities with well-characterized OXPHOS pathologies, particularly with Leber's hereditary optic neuropathy (LHON), caused by mutations in mtDNA-encoded genes for subunits of complex I of the respiratory chain (Larsson et al., 1991). Whether OPA1 dysfunction however leads to a reduction in mtDNA is still debated. The reduction in ADOA patients was reported, while an analysis in the mouse models for ADOA did not show any difference in the mtDNA number (Alavi et al., 2006).

Why, despite OPA1 being expressed ubiquitously, ADOA manifests itself with a clinical predominant ocular phenotype remains unclear however. Although OPA1 protein levels in the retina are particular high (Alexander et al., 2000) they are not higher in RGCs than in any other retinal cells (Kamei et al., 2005). RGCs however have a particularly high energy requirement since the initial portion of their axon, that heads from the optic nerve head to the lamina cribrosa is unmyelinated and very rich in mitochondria to provide ATP for visual signal transmission (Carelli et al., 2004). Hence, the high demand of energy in RGCs may account for the defect caused by OPA1 dysfunction.

In conclusion, despite in depth genetic analyses, the mechanism underlying ADOA still remains to be elucidated. Data however suggests that there may be several mechanisms dependent on the mutation leading either to haplosufficiency or missense mutations.

Aims of the Thesis

The multifunctional GTPase OPA1 functions in numerous crucial aspects of mitochondrial physiology including mitochondrial fusion, apoptosis, and oxidative phosphorylation. Despite our growing knowledge of the biological mechanisms in which OPA1 operates since its discovery in 2000, fundamental gaps in our understanding of OPA1 function remain.

This PhD project aimed at deepening our understanding of the mechanisms underlying OPA1 function by characterizing alterations in GTPase activity and protein behavior caused by ADOA mutations in OPA1. Moreover, we aimed to address the possibility of targeting the catalytic domain of OPA1 with a small compound inhibitor.

To that end, the specific aims of this thesis were:

(I.)   Biochemical and genetic characterization of the effect of ADOA mutations on OPA1 GTPase-dependent behavior *in vitro* and *in vivo*.

(II.)   Development and implementation of a semi-automated *in vitro* high-throughput screen of a small compound library to identify chemical drugs that inhibit OPA1 GTPase activity.

Results

(I.)   Opa1 mutations causing Autosomal Dominant Optic Atrophy plus act as
        dominant negative because they cause negative enzyme cooperativity.

(II.)   An *in vitro* high throughput-screening assay identifies small compounds
        that inhibit the antiapoptotic mitochondrial GTPase OPA1.

Opa1 mutations causing Autosomal Dominant Optic Atrophy plus act as dominant negative because they cause negative enzyme cooperativity

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Summary

A milder "classic" and a more severe "plus" form of autosomal dominant optic atrophy (ADOA) both caused by mutations in the mitochondrial GTPase Optic atrophy 1 (Opa1) exist, but why certain mutations result in classic while others in plus ADOA forms is unclear. Here we demonstrate that the difference between ADOA classic and plus mutations relies on how they affect the cooperativity of OPA1 GTPase function. In a yeast system engineered to test Opa1 pathogenicity, ADOA plus but not ADOA classic mutations impaired function of the wild type protein in respiration and mitochondrial fusion. Biochemically, while GTPase activity of recombinant Opa1 was reduced by all ADOA classic and plus mutations tested, only the ADOA classic mutations impaired Opa1 cooperativity, explaining why they do not act as dominant negative. In conclusion, cooperative Opa1 catalysis is a major feature in determining the clinical outcome of ADOA mutations.

Introduction

Autosomal dominant optic atrophy ADOA is the most frequent form of hereditary optic neuropathy (Kjer et al., 1996). Clinically, it is characterized by vision loss, vision field defects, tritanopia and optic nerve pallor caused by the selective degeneration of retinal ganglion cells and optic nerve atrophy (Kjer et al., 1996; Lenaers et al., 2012). The majority of ADOA cases originate from mutations in the mitochondrial GTPase optic atrohy 1 (*OPA1*) (Alexander et al., 2000; Delettre et al., 2000) but also other gene mutations have been implicated in the development of the disease (Waterham et al., 2007; Zuchner et al., 2006).

OPA1 is a nuclear gene composed of 30 coding exons of which exons 4, 4b, and 5b are alternatively spliced generating 8 mRNA variants (Delettre et al., 2001). Its protein product Opa1 localizes to the inner mitochondrial membrane facing the intermembrane space. Opa1 is proteolytically processed by the proteases Yme1l in normal conditions or Oma1 in stress conditions, yielding short forms (Head et al., 2009; Song et al., 2007). Opa1 belongs to the family of dynamin like large GTPases that control mitochondrial morphology and together with the outer mitochondrial membrane protein mitofusin (Mfn) 1 it mediates mitochondrial fusion (Cipolat et al., 2004). Like the other members of this dynamin superfamily, Opa1 contains a highly conserved GTPase domain and a C-terminal GTPase effector domain (GED), which mediate and presumably regulate its catalytic activity (Ford et al., 2011; Hanna Damke, 2001). Opa1 is the human orthologue of *S. cerevisiae* mitochondrial genome maintenance 1 (Mgm1), first characterised in a genetic screening as a dynamin-like protein involved in the propagation mtDNA. Although the amino acid sequence of OPA1 and Mgm1 are only by 17% identically they share the basic protein build up (Delettre et al., 2000) and participate in similar cellular functions. Like Opa1 also Mgm1p localises to the IMM and is involved in coordinating the sequential fusion of the outer and inner mitochondrial membranes together with Fzo1 and Ugo1 (Wong et al., 2003). It is processed into two isoforms: a long form (l-Mgm1), inserted in the IMM facing the IMS

and a short form (s-Mgm1), peripherally attached to the IMM (Herlan et al., 2003). The short form of Mgm1 is generated by proteolytic processing of the long form at the rhomboid cleavage region RCR by Pcp1p, the homologue of mammalian PARL (Herlan et al., 2003). Notably, an equal amount of both the forms is needed to the yeast cells to be respiratory competent and for mitochondrial fusion to occur: while s-Mgm1 possesses the GTPase activity and mediates the fusion activity, l-Mgm1 serves as a scaffold for the short form to exert its function (Zick et al., 2009).

In addition to its function in mitochondrial fusion, Opa1 controls mitochondrial ultrastructure and impinges on the cristae remodelling pathway to regulate cytochrome *c* redistribution from the intracristal to the intermembrane space (Scorrano et al., 2002) and therefore apoptosis independently from its function in mitochondrial fusion but requiring an intact GTPase and C-terminal GED (Cipolat et al., 2006; Frezza et al., 2006). Additionally, by regulating mitochondrial ultrastructure Opa1 favours the assembly and stability of respiratory chain supercomplexes and enhances mitochondrial respiration efficiency (Cogliati et al., 2013; Frezza et al., 2006). The antiapoptotic and metabolic effects of Opa1 cooperate in protecting tissues from atrophic, apoptotic and necrotic damage and in correcting mouse models of primary mitochondrial dysfunction (Civiletto et al., 2015; Varanita et al., 2015). These cell biology studies however failed to definitively clarify how *OPA1* impair Opa1 function and cause ADOA.

Progression and severity of OPA1-related ADOA is highly heterogeneous. In addition to the more benign ADOA classic in which symptoms are restricted to visual loss, a more severe form termed ADOA plus was identified. ADOA plus describes a multi-systemic disorder with vision defects, moderate sensorineural hearing loss, peripheral neuropathy, ataxia and myopathy (Amati-Bonneau et al., 2003). Many of these symptoms are associated with mtDNA instability and impairments of mitochondria-located oxidative phosphorylation (OXPHOS) resembling the clinical presentation of classical OXPHOS diseases (Amati-Bonneau et al., 2008; Hudson et al., 2008; Zeviani, 2008) A widely accepted model postulates that haploinsufficiency is the cause of classic ADOA, given that 50% of mutations result in

a truncated protein and deletion of the whole genetic locus of OPA1 clinically presents as ADOA. Conversely, missense *OPA1* mutations concentrate in the GTPase domain and they might cause ADOA plus phenotypes by exerting a dominant negative effect (Amati-Bonneau et al., 2008; Olichon et al., 2007) as also suggested by the dominant negative effect of GTPase mutants of Mgm1 in yeast containing a wild type (WT) allele of the dynamin (Guillou et al., 2005; Shepard and Yaffe, 1999; Wong et al., 2003). However, this model is challenged by the retrieval of several classic ADOA mutations lying in the GTPase or in the GED domain (R290, S353, A412, R879) (Ferre et al., 2005). The genotype phenotype correlation is further complicated by the current ignorance of the kinetics of GTP hydrolysis by Opa1 and by the lack of a quick model where the effect of mutated *H. sapiens* Opa1 can be tested on mitochondrial morphology and function. With this in mind, we used a two-pronged approach by combining a *S. cerevisiae* model engineered to express *H. sapiens OPA1* and kinetic analyses on recombinant wild type and mutated Opa1. Our results identify catalytic cooperativity as a key determinant of classic or plus ADOA phenotype.

Results

Deletion of Mgm1 is rescued by expression of a Mgm1-OPA1 chimera

Opa1 in *H. sapiens* and Mgm1 in *S. cerevisiae* display similar functions in inner mitochondrial membrane fusion (Cipolat et al., 2004; Guan et al., 1993), cristae morphology (Amutha et al., 2004; Frezza et al., 2006) mtDNA maintenance (Hudson et al., 2008; Jones and Fangman, 1992) and oxidative phosphorylation (Cogliati et al., 2013; Guan et al., 1993). We therefore decided to verify if human *OPA1* can compensate for the deletion of Mgm1 in *S. cerevisiae*. Complete disruption of *Mgm1* in *S. cerevisiae* results in mtDNA loss and the arising *petite* phenotype cannot be rescued by subsequent expression of the WT gene (Guan et al., 1993). However, since Mgm1 and Opa1 amino acid sequence share only 17% of identity, most residues hit by ADOA associated mutations are not retrieved in Mgm1, calling for a system where human Opa1 is reintroduced in *S. cerevisiae*. Given that conventional reintroduction of Mgm1 in a Δ*Mgm1* background fails to rescue the deleted phenotype, we turned to the well-established tetrads dissection approach to delete one or both Mgm1 alleles and to replace them with each of the 8 *OPA1* splice variants. Following sporulation we selected haploid yeast strains carrying the deletion of chromosomal *Mgm1* and the individual *OPA1* isoform or reintroduced *Mgm1* on a centromeric plasmid. Chromosomal *Mgm1* gene deletion impaired yeast growth on non-fermentable medium. The defect was specific, as indicated by its complete rescue upon centromeric reintroduction of WT Mgm1, but was not recovered by expression of the individual human *OPA1* splicing variants (Figure 1A). Because all human isoforms are expressed at comparable levels and cleaved in the Δ*Mgm1* strain (data not shown), instability of OPA1 splicing variants could not explain the observed lack of complementation.

Since the processing of OPA1 and Mgm1 differs (Duvezin-Caubet et al., 2007), we hypothesized that this different cleavage mechanism could explain the lack of Δ*Mgm1*

complementation by Opa1. We therefore generated a Mgm1-Opa1 chimeric protein where the transmembrane region of Opa1 was replaced by the Mgm1 region containing the transmembrane domain and the rhomboid cleavage region (RCR) recognized by the yeast processing protease Pcp1p (Herlan et al., 2003) (CHIM2, Figure 1B). The chimeric protein was correctly expressed (albeit to a lower level) and cleaved into a short form with a molecular weight comparable to that of WT Mgm1p (albeit with lower efficiency, Figure 1C). Accordingly, expression of the Mgm1-Opa1 chimera partially rescued ΔMgm1 mitochondrial morphology (Figure 1D, F), mtDNA content (Supplementary Figure S1C) and growth on non-fermentable medium (Figure 1E). The decreased complementation was not caused by the difference between yeast and mammalian mitochondrial targeting sequence: a chimera where the targeting sequence was also derived from Mgm1 (CHIM1, Supplementary Figure S1A) complemented the ΔMgm1 phenotypes to the same extent of CHIM2 (Figure 1D-F and Supplementary Figure S1B). We therefore suspect that the slight reduction in Δ*Mgm1* complementation by Mgm1-Opa1 chimera is a consequence of its lower expression and less efficient cleavage. In conclusion, we engineered a yeast model that allows studying the impact of Opa1 mutations on mitochondrial and cellular functions.

Intact GED and GTPase domains of the Mgm1-Opa1 chimera are required to complement a ΔMgm1 yeast strain

We capitalized on our engineered yeast model to verify pathogenicity and mechanism of action of ADOA associated mutations. We selected two mutations reported to cause ADOA plus: R445H in the GTPase domain and V910D in the GTPase effector domain, as well as the K301A mutation in a highly conserved Lys residue in the GTPase domain P loop, reported to act as dominant negative when coexpressed with the WT protein, and a mutation associated with ADOA classic: G300E in the GTPase domain (Table 1 summarizes the different mutations tested). Moreover, we tested several mutations in the mitochondrial import sequence: Q15K, delS38R43 and Y80C; and Y102C in the transmembrane domain. A ΔMgm1 haploid strain carrying the WT gene associated to the URA3 marker was therefore

engineered to express the WT and mutated Mgm1-Opa1 chimeras from a centromeric vector with the TRP1 marker.

Immunoblot analysis confirmed that the mutated chimeras were all competently expressed at comparable levels and revealed that except for the V910D mutation in the GTPase effector domain which causes a slight accumulation of the short form, none of the tested mutations affect processing of the Mgm1-Opa1 chimera (Figure 2A). These results indicate that the mitochondrial targeting sequence mutations do not impair import at least in yeast. We then grew cells on minimal medium without tryptophan to select strains expressing only the mutated CHIM2 carrying the TRP1 marker and to verify their effect on mitochondrial and cellular function (Figure 2B). As expected, the Mgm1-Opa1 chimera fully corrected the ΔMgm1 growth defect on non-fermentable carbon source (Figure 2B). Similarly, growth was restored by chimeras carrying mutations in the import sequence and to a lower level by the chimera carrying the Y102C mutation in the transmembrane domain (Figure 2B). However, all chimeras carrying mutations in the GTPase domain and GTPase effector domain failed to complement the ΔMgm1 strain, irrespective of whether they are associated with ADOA classic or ADOA plus (Figure 2B). These results indicate that intact GTPase and GED domains are required for Opa1 function but fail to explain once again the genotype-phenotype correlation between Opa1 mutations and ADOA types.

GTPase domain mutations exert a dominant negative effect on wild type Mgm1

Since we could not find a definitive correlation between ADOA classic, ADOA plus mutation and complementation of the ΔMgm1 strain growth defect on non fermentable carbon source containing media, we set out to explore whether the mutations tested here displayed a dominant negative effect. To this end, we decided to coexpress mutated and WT Mgm1-Opa1 chimeras in ΔMgm1 yeast. Coexpression of WT or Q15K, delS38R43, Y80C, Y102C and V910D Mgm1-Opa1 chimeras together with WT Mgm1-Opa1 partially rescued the respiratory phenotype of ΔMgm1 strains. Conversely, coexpressing the GTPase domain

mutant proteins G300E, K301A, and R445H reduced the growth on a non-fermentable carbon source (Figure 3A), suggesting that these mutations impair function of WT Mgm1-Opa1. Indeed, the GTPase K301A and R445H mutants caused mitochondrial fragmentation when coexpressed with WT Mgm1-Opa1, whereas mitochondrial morphology of the strain coexpressing WT Mgm1-Opa1 with the GED mutant V910D or with the GTPase G300E mutation was comparable to that of yeast expressing two alleles of WT Mgm1-Opa1 (Figure 3B, C). In conclusion, ADOA plus but not ADOA classic mutations exert a dominant negative effect on endogenous protein, via an uncharacterized mechanism.

ADOA mutations decrease Opa1 cooperativity

It seems plausible to consider that a dominant negative effect may be related to the enzymatic function of Opa1, but our knowledge on the enzymatic function of this large GTPase is very scarce. Hence, we established a protocol to purify a recombinant form of soluble Opa1 (lacking the import sequence and the transmembrane domain, Figure 4A) by adding a 6-His tag to the C-terminus of the protein. Induction of soluble Opa1 expression in competent BL21-CodonPlus (DE3)-RIPL *E. coli* bacteria yielded 55 µg of soluble protein per litre of bacterial culture, which we proceeded to purify by affinity chromatography on Ni-NTA beads. Since a contaminant protein with intrinsic ATPase activity co-purified with recombinant soluble Opa1, we removed it by extensive washes with ATP and we eluted by increasing imidazole concentrations (Supplementary Table S1) obtaining moderate yields of recombinant pure soluble Opa1 (rOpa1, Fig. 4B) devoid of contaminating ATPase activity that might confound GTPase measurements (not shown). Enzymatic activity of the dialyzed rOpa1 was first determined by reverse phase chromatography (RPC). Results were fully superimposable to those obtained by the colorimetric malachite green assay which quantifies the free phosphate released during the hydrolysis of GTP into GDP (Leonard et al., 2005) (Fig. 4C and Table 2). As the case for other dynamin-like GTPases, Opa1 required Mg^{2+} as cofactor for GTP hydrolysis (Supplementary Figure S2A, B), which was

irreplaceable by $MnCl_2$ (Supplementary Figure S2C, D). GTPase activity was not influenced by 500 µM GDP, excluding inhibition by GDP throughout the period of the GTPase assay (Supplementary Figure S2E). While increasing concentrations of KCl up to 100 mM only slightly decreased rOpa1 GTPase activity, 100 mM NaCl reduced it by 80% (Supplementary Figure S2). Hence, all our measurements were carried out in a low ionic strength buffer containing 10 mM $MgCl_2$. The Michaelis constant (K_m) value 106 µM for rOpa1 lies in the reported range of other dynamins (between 10 and 100 µM (Song and Schmid, 2003). Fitting a Hill equation curve to the rate data at different substrate concentrations reveals a sigmoidal behavior at low substrate concentrations, with a Hill coefficient above 1 (Figure 5A, C, Table 2). This Hill coefficient indicates positive cooperativity and suggests that rOpa1 activity is enhanced after the protein binds its substrate, like all dynamin related GTPases (Song and Schmid, 2003). To verify if also Opa1 oligomerizes, as previously indicated by experiments where Opa1 (or Mgm1p) oligomers were retrieved in mitochondria (Cipolat et al., 2006; Cogliati et al., 2013; Frezza et al., 2006; Meeusen et al., 2006) we performed a crosslinking experiment using glutaraldehyde vapor diffusion (Fadouloglou et al., 2008) to covalently crosslinks rOpa1 protein complexes (Lusty, 1999). SDS-PAGE revealed that upon glutaraldehyde treatment rOpa1 quickly forms dimers followed by tetramers, correlating with the disappearance of monomers. By 10 minutes monomers have completely disappeared to form dimers and tetramers and at 20 minutes the majority of protein is found in tetramers (Figure 4D). In conclusion, rOpa1 displays a cooperative catalysis, which correlates with the assembly in higher order oligomeric structures. These results represent a conceptual framework to test whether and how the studied mutations retrieved in patients affect Opa1 catalytic activity.

We produced rOpa1 carrying the G300E (ADOA classic), R445H (ADOA plus), K301A (putative dominant negative) and V901D (ADOA plus) mutations with yields comparable to that of WT rOpa1. The produced proteins displayed also comparable stability *in vitro* and did not aggregate as indicated by light scattering (not shown). We therefore decided

to compare their enzymatic properties using the malachite green GTPase assay. All tested mutations increased rOpa1 Michaelis constant K_m value, indicating that they decrease the affinity for GTP. Maximal velocity V_{max} and enzyme turnover k_{cat} were increased in rOpa1^{K301A} and decreased in rOpa1^{R445H}, whereas they were not affected by the G300E and V910D mutations (Figure 5A, C and Table 3). The catalytic efficiency ratio (k_{cat}/K_m) was slightly reduced by the ADOA classic mutation G300E and the potential dominant negative K301A mutation, whereas it was reduced by more than 50% by the ADOA plus V910D and R445H mutations. When we turned into measurements of Hill coefficients, we found that the coefficient was unaffected in the dominant negative rOpa1^{K301A} and in the ADOA plus rOpa1^{R445H} mutants, whereas it was reduced to 0.85 (implying negative cooperativity) in rOpa1^{G300E} and to approximately 1 (indicating no cooperativity) in rOpa1^{V910D}. We therefore identified two different types of enzymatic alterations caused by Opa1 mutations: one characterized by defective GTPase activity due to a decrease in affinity to GTP, possibly due to a loss of cooperativity (G300E and V910D) and a second by affected GTP turnover with maintained cooperativity (K301A, R445H). In line with these enzyme kinetics results, oligomeric assembly of rOpa1^{G300E} and rOpa1^{V910D} but not of rOpa1^{K301A} was impaired (Figure 5B, D), further supporting cooperativity as a key predictor of clinical outcome of Opa1 mutations. In conclusion, our classification indicates that Opa1 mutations present clinically as ADOA classic because they impair cooperativity and lends a molecular explanation to the clinical feature and the yeast phenotypes observed here.

Discussion

Clinical presentation of ADOA is highly variable, and a clear genotype phenotype correlation lacks. Here we provide evidence that cooperativity is a key feature of the protein behavior, indicative of whether an *OPA1* mutation results in ADOA classic or plus clinical presentation.

Understanding the genotype-phenotype correlation in ADOA is complicated by the lack of an appropriate system to compare the impact of different mutations on Opa1 and cellular function. Although mouse models for ADOA have been generated (Alavi et al., 2006), they do not allow a quick comparison of different mutations. Conversely, using yeast as a model to validate the outcome of single Opa1 point mutations in principle bears several advantages, ranging from its rapidity to the obtainable clear readout. However, Opa1 is only 17% identical to its yeast orthologue Mgm1p, complicating to model human mutations on such a divergent protein and calling for a hybrid system where *S. cerevisae* lacking Mgm1 are complemented with Opa1. When we engineered such a model we observed that Opa1 did not complement ΔMgm1 strain. We excluded that the issue was caused by different import properties of the mammalian protein in the yeast mitochondria and we mapped it to the differential cleavage of the protein. All the introduced Opa1 isoforms underwent one or more cleavage in yeast (not shown), but none of them was able to recover growth on non fermentable carbon sources, indicating that they were unable to correct ΔMgm1 strain mitochondrial dysfunction. An engineered chimera containing Mgm1p transmembrane domain and cleavage site can conversely correct the growth and mitochondrial morphology defect of ΔMgm1 strain. Our data formally demonstrate that Opa1 and Mgm1p are orthologues and that they diverge in the function of the transmembrane and cleavage sites domains, as it was already suggested by studies in yeast where lack of the rhomboid protease Pcp1p did not affect Opa1 cleavage (Duvezin-Caubet et al., 2007). However, since recognition by the rhomboid protease might depend on Opa1/Mgm1 regions distant from

the cleavage site, our engineered Mgm1-Opa1 chimera demonstrates that the divergent cleavage of Opa1 and Mgm1p depends directly on the rhomboid cleavage region (Schäfer et al., 2010).

The Mgm1-Opa1 chimera was instrumental to verify the effect of ADOA mutations with different clinical presentations (ADOA classic or plus) on mitochondrial and cellular function. Of note, the mutations located within the GTPase domain and the effector domain failed to rescue growth of a ΔMgm1 strain, whereas mutations in the import sequence and in the transmembrane domain did. While these experiments substantiate the essential role of Opa1 GTPase activity for its function, they fail to establish a clear genotype-phenotype correlation: indeed, the G300E and the R445H mutants display a similar behavior, but they are associated with ADOA classic and plus clinical pictures. When we coexpressed WT and mutated Mgm1-Opa1 chimeras in a ΔMgm1 background we could determine that the K301A, the R445H but not the import sequence, transmembrane domain or G300E and V910D mutations acted as dominant negative. This picture was consistent with the clinical presentation of ADOA plus caused by the R445H mutation, yet it failed to explain how mutations in two adjacent residues (G300 and K301) could lead to completely different phenotypes and called for a detailed analysis of Opa1 enzymatic function.

While several studies addressed Mgm1 enzymatic activity (DeVay et al., 2009; Meeusen et al., 2004; Meglei and McQuibban, 2009) our knowledge on Opa1 kinetics is conversely scarce. A short recombinant Opa1 protein displays all the features of a classical dynamin related protein and a V_{max} that lies in the range reported for other dynamin-like GTPases (Song and Schmid, 2003). Mgm1p oligomers display increased GTPase activity (Meglei and McQuibban, 2009), confirming that oligomerization increases activity of this dynamin related protein and suggesting that Opa1 also displays a cooperative catalytic mechanism. Indeed, Hill coefficient was >1 for Opa1, indicating that oligomerization of the protein favors GTP hydrolysis. Consistently, recombinant Opa1 assembled in dimers and tetramers and mutations in Opa1 differentially affected its cooperative behavior. Surprisingly however, cooperativity is impaired by ADOA classic mutations whereas it is not affected by ADOA

plus mutations. The loss of cooperativity by the classic mutations may reduce their capacity to cooperate with the wildtype protein and hence leave it unaffected. Indeed, these mutations affect higher order assembly of recombinant Opa1. Our results explain how mutations that are not leading to truncated proteins act as haploinsufficient, the major pathomechanism underlying ADOA classic (Alavi et al., 2006; Alexander et al., 2000; Fuhrmann et al., 2014): these mutations, like G300E impair GTPase activity and at the same time reduce cooperativity. Our conceptual framework perfectly predicts the behavior of the V910D mutation, originally classified as ADOA plus but resulting in milder symptoms essentially resembling those of ADOA classic (Amati-Bonneau et al., 2008). Indeed, Hill coefficient of the V910D Opa1 mutant is close to 1, implying loss of cooperativity as also reflected by the impaired higher order Opa1 assembly.

Strikingly, mutations in the neighbor amino acids G300 and K301 cause different effects on OPA1 function *in vitro* as well as *in vivo*: while the former reduces cooperativity, impairs Opa1 oligomerization and does not act as dominant negative, the latter leaves cooperativity unaltered, does not affect Opa1 oligomerization and inhibits function of endogenous wildtype Opa1. This is in line with the role of K301 in Opa1 dimerization hypothesized by analogy with the role of the corresponding Lys in dynamin-1 (Sundborger et al., 2014): its mutation generates a dominant negative form of the dynamin (Damke et al., 1994) because it stabilizes it in an oligomeric prefission form (Sundborger et al., 2014). Similar to the Opa1 K301A mutant, also the dynamin K44A mutant hydrolyses GTP more efficiently presumably because its oligomers are more resistant to GTP induced disassembly (Warnock et al., 1996).

In vivo analyses of OPA1 indicate that it oligomerizes to participate in multi-protein high molecular weight complexes, which are crucial for cristae shape (Frezza et al., 2006). Disruption of these oligomers abolishes the antiapoptotic function of OPA1 due to facilitated cytochrome *c* release (Frezza et al., 2006) and reduces respiratory efficiency (Cogliati et al., 2013). In line with the here presented dimer and tetramer formation it is plausible to consider that OPA1 GTP hydrolysis is oligomerization dependent.

In summary our two-pronged approach not only characterized several basic features of

enzymatic function of Opa1, but it also identified cooperativity as a key factor in determining the consequence of an ADOA mutation on mitochondrial and cellular function, explaining how two adjacent mutations can result in completely different phenotypes. The engineered yeast strain described here can therefore be used faithfully to test novel *OPA1* mutations for pathogenicity and to predict clinical features.

Materials and Methods

Molecular Biology

OPA1 human isoforms 1, 2, 4, 5, 6, and 7 were amplified from constructs available in the laboratory cloned into the pCR8/GW/TOPO TA entry vector Invitrogen according to manufacture's protocol.

Human OPA1 isoform 3, containing exons 4b and 5 was obtained by an external PCR from pCR8::hOPA1 isoform 6, containing exons 4b, 5 and 5b. The PCR product was then re-ligated with the T4 DNA ligase Promega and cloned inside the pCR8/GW/TOPO TA entry vector Invitrogen as above. Human OPA1 isoform 8 was obtained by cloning a fragment containing the exons from 2 to 5b synthesized by Genescript in the pUC57 vector into pCR8::hOPA1 isoform 4. The two vectors were digested with SnaBI and SacI restriction endonucleases New England Biolabs. The shorter fragment from pUC57::hOPA1 exons 2-5b plasmid and the longest one from pCR8::hOPA1 isoform 4 were isolated by DNA gel electrophoresis, extracted and purified using the QIAquick Gel Extraction Kit Qiagen and then ligated together with the T4 DNA ligase Promega.

Yeast Mgm1 gene was amplified from yeast genomic DNA and cloned inside the pCR8/GW/TOPO TA entry vector Invitrogen as above.

CHIM1 construct, including the MTS and the TM domain of Mgm1 from +1 to +582 of Mgm1 ORF), fused to the c-terminal of OPA1 from +517 to stop of isoform 2 ORF was obtained by PCR overlapping. The n-terminal portion of Mgm1 was amplified from pCR8::Mgm1, while the C-terminal portion of OPA1 was amplified from pCR8::hOPA1 isoform 2. Both Mgm1-OPA1 primers have the 5' part that overlaps with the opposite fragment, allowing them to recombine. After purification, a mix of the two PCR fragments was subjected to another PCR cycle to obtain the hybrid construct. The resulting product was inserted into the pCR8/GW/TOPO TA entry vector Invitrogen. CHIM2, containing

only the TM domain of Mgm1 from +278bp to +582 of Mgm1 ORF fused to the MTS of OPA1 at the N-terminal from 1bp to 448bp and to the C-terminal part of OPA1 from +517 to stop of isoform 2 ORF), was obtained via PCR overlapping at the same conditions described above, using pCR8::hOPA1 isoform 2 and pCR8::CHIM1 as templates.

The human OPA1 splicing variants, the Mgm1-OPA1 hybrid form and the Mgm1 variant genes were then cloned into the pCM189-RfB destination vector, containing the URA3 selection marker, or the pCM184-RfB destination vector, containing the TRP1 selection marker, via homologous recombination using the Gateway LR Clonase II enzyme mix Invitrogen according to manufacturer's protocol. pCM189-RfB and pCM184-RfB were obtained by cloning the RfB cassette Invitrogen into the PmeI site of the two pCM189 and pCM184 vectors.

To produce recombinant OPA1 the mouse transcript variant 2 of OPA1 was amplified from basepair 502 to the stop of the ORF. The PCR product was cloned into the pET21+ vector, which adds a sequence encoding for six aligned histidines to the C-terminus of the encoded protein Novagen. Plasmids encoding the mutant proteins were generated by site-directed mutagenesis by PCR-amplification of the wildtype construct with primers carrying the desired mutation.

Yeast strain and media

The experiments were performed using a W303 strain Mat a/Mat alpha; leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15. Yeast strains were cultured in rich media YP or synthetic minimal medium SM at 30°C, as described (Burke and Kwast, 2000). YPDA 1% yeast extract, 2% peptone, 2% dextrose was used to routinely maintain wild type and ΔMgm1 strains. Synthetic minimal medium 0.17% yeast nitrogen base without amino acids, ammonium sulphate 0,5%, glucose 2% contained the necessary auxotrophic supplements was used to select the transformant strains. For FOA selection, synthetic minimal medium containing uracil and the other essential auxotrophies was supplemented with 100X

5-fluoroorotic acid solution Zymoresearch according to manufacturer protocols. YPGly 1% yeast extract, 2% peptone, 3% glycerol was used to test the respiratory ability of the mutant strains. SMP sporulation medium, 1% potassium acetate was used to induce the sporulation of diploid yeast strains. 2% agar was added to the solid media.

Mgm1 deletion

Deletion of Mgm1 gene was obtained by replacing the Mgm1 genomic sequence with the KANMX4 gene sequence on a W303 diploid strain. The KANMX4 coding sequence, that confers the kanamicyn resistance, was amplified from pFA6a-kanMX4 vector (Wach et al., 1994). The primers contained 45 bp of homology with the 5' and 3' of Mgm1 ORF to allow homologous recombination between the PCR fragment and the Mgm1 genomic region. The PCR product was transformed into the diploid strain using a high efficiency lithium acetate method as previously described (Gietz and Woods, 2006). Colonies with the insertion of the cassette were selected by growth in rich medium with 400 µM geneticin, and the correctness of the insertion was proved by PCR after genomic DNA extraction. Only colonies with heterozygous deletion of Mgm1 were selected for further studies.

Yeast transformation

Transformations of the diploid strain with the pCM184 and pCM189 vectors were performed with the PEG-Lithium acetate method as previously described (Gietz and Woods, 2006).

Tetrads dissection and analysis

Heterozygous ΔMgm1 mutants were let to sporulate on solid SPM medium for 4 days at 25°C. About 10 tetrads per strain were mechanically dissected as described (Sartori et al.,

2000) and the spores were incubated on YPDA at 30°C for 3 days. For each spore, the correct 2:2 segregation of the kanamycin resistance and of the different auxotrophic markers was verified by replica-plate on selective media. Haploid cells deleted for Mgm1 and auxotrophic for the vector marker were used for subsequent studies.

FOA treatment

Treatment with 5-fluoroorotic acid 5-FOA), a compound that is converted into the toxic fluorouracil in the strain expressing the URA3 gene, was used to select yeast cells expressing CHIM2 mutant alleles. The haploid ΔMgm1 carrying pCM189::CHIM2 wild type obtained by the tetrad dissection was transformed with pCM184::CHIM2 wild type and mutated constructs. Transformed cells were selected in minimal medium without tryptophan and uracil SM GLU-URA-TRP. For each mutant, three colonies were grown in liquid culture and plated on minimal medium without tryptophan supplemented with FOA 1mg/ml. Grown colonies were propagated in minimal medium without tryptophan SMGLU-TRP and used for further analysis.

Protein and DNA extraction

Yeast cultures were grown in synthetic minimal medium lacking the uracil or tryptophan until they reached stationary phase. For protein extraction, 1 ml of cells was harvested and mechanically lysed using standard rapid glass-bead lysis in a MagnaLyzer instrument Roche Diagnostic. Proteins were precipitated with trichloroacetic acid and resuspended in Laemmli loading buffer (Laemmli, 1970). Equal aliquots of protein extracts were resolved by SDS-PAGE. DNA was extracted from 1ml of stationary phase cells culture using standard rapid glass-bead lysis and phenol-chloroform purification using standard protocol.

Analysis of protein expression

Equal amounts of total cell extracts were were resolved by 7% Tris Glycine SDS-PAGE Biorad. Fractions of the affinity purification of recombinant Opa1 were separated on a 4-12% Bis-Tris SDS-PAGE Invitrogen. Subsequent western blotting was perfomed using monoclonal mouse anti-OPA1 diluited 1:1000 BD Transduction Laboratories or polyclonal rabbit anti-Mgm1 antibody diluited 1:1000, followed by peroxidase-labeled goat anti-mouse or anti-rabbit respectively secondary antibody diluited 1:5000 allowed detection by enhanced chemiluminescence Amersham Pharmacia Biosciences. Alternatively, proteins on SDS-PAGE gels were visualized with Coomassie. The Mgm1 antibody was a kind gift from Dr. Michael Zick, Munich University, Germany.

Analysis of mitochondrial morphology

Mitochondrial morphology was assessed by fluorescence microscopy as previously described (Dimmer et al., 2002). Briefly, yeast cells were transformed with pYX142-mtGFP construct, carrying a mitochondrial targeted GFP (Westermann and Neupert, 2000). After growth to logarithmic phase in selective glucose medium yeast cells were resuspended in 1% w/v in PBS low melting agarose at 37° and subjected to standard fluorescence and phase contrast microscopy by using the AXIOSKOP 2 plus system Zeiss and ProgRes MF camera JENOPTIK equipped with 100x oil objective. At least 80 cells for three independent experiments were analyzed and classified according to the presentation as elongated and spread on all cell surface mitochondria spread-elongated or short and clustered mitochondria clustered-fragmented. pYX142-mtGFP was a kind gift by Doctor Kai Stefan Dimmer.

mtDNA level measurement

MtDNA levels were measured using Quantitative Real time PCR in the Rotor-Gene 6000 Corbett Research and analyzed as previously described (Taylor and Turnbull, 2005) using the Rotor-Gene 6000 Series Software 1.7.

Recombinant protein

Truncated recombinant transcript variant 2 of OPA1 (NM_133752) lacking the N-terminal transmembrane domain was expressed in *Escherichia (E.) coli*. Protein production was induced with 0.5 μM IPTG for 20 hours at 18°C. *E. coli* were subsequently collected by centrifugation and the pellet was resuspendend in lysis buffer 40 mM Hepes/KOH, 500 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5% Triton-X-100, 2% Tween-20, 1 mM PMSF, 20 mM Imidazole at pH 8.0. To prevent proteolysis an EDTA-free proteinase inhibitor cocktail Roche was added. Cells were lysed by sonication and cell debris was removed by centrifugation at 14 000 x g for 45 minutes at 4°C. Recombinant OPA1 was subsequently purified based on a C-terminal 6 x histidine tag by Ni-NTA batch chromatography. Prior to elutions the protein bound beads were washed with 1 mM ATP and 10 mM $MgCl_2$ in lysis buffer for 30 minutes at room temperature followed by an intermediate washing step in wash buffer 40 mM Hepes/KOH, 300 mM NaCl, 0.05% Triton-X-100, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% glycerol, 20 mM Imidazole at pH 8.0. Subsequently, OPA1 was eluted with increasing concentrations of imidazole in elution buffer 40 mM Hepes/KOH, 0.05% Triton-X-100, 5 mM β-mercaptoethanol, 10% glycerol at pH 7.4. Imidazole was removed by buffer exchange and the protein was concentrated into storage buffer 40 mM Hepes/KOH, 0.05% Triton-X-100, 0.3 mM TCEP, 10% glycerol and stored at -80°C until use.

GTPase Activity

GTPase activity was measured using an assay based on the change of absorbance of malachite green in the presence of free PO_4 ions that results from the hydrolysis of GTP into GDP (Leonard et al., 2005). A mixture containing containing 8 mM ammonium molybdate and 1.5 mM malachite green was freshly prepared the day of use. The change in absorbance molybdate and the free phosphate form a phosphomolybdate complex (Cogan et al., 1999) that then binds the malachite green dye resulting in a change of absorbance depending on the contained phosphate concentration that was measured at 595 nm after a 15 minutes incubation period at room temperature. Phosphate concentrations were subsequently extrapolated from a P_i standard curve ranging from 0 to 100 μM.

Alternatively, a reverse phase chromatography based method was used. Equally in a final reaction volume of 20 μL recombinant OPA1 was mixed with 500 μM GTP and 10 mM $MgCl_2$ and kept at 37°C. At the respective timepoints, the hydrolysis reaction was stopped by boiling 20 μL of the sample at 99°C for 10 minutes. The sample was then spun for 10 minutes at 13 000 x g and the supernatant was stored at -20°C until processing. To analyze the GDP content the sample was loaded on a reverse phase column Synergi 4n MAX-RP 80A in 50 mM sodium phosphate buffer containing 10 mM tetrabutylammonium bromide and 4% acetonitrile at a final pH of 6.0 on a high-pressure liquid chromatography apparatus to separate the contained GTP from GDP. Their final molar concentrations were determined using the Lambert-Beer law with their extinction coefficient of 13 700 $M^{-1}cm^{-1}$.

Crosslinking

Recombinant OPA1 was crosslinked using the glutaraldehyde vapor-diffusion method as described by Fadouloglou et al. 2008. In brief, 50 μL of rOPA1 at a concentration of 0.9 μM were placed on a microbridge in a well of a 24-well plate containing 400 μL of

glutaraldehyde solution Sigma. The well was sealed using vacuum grease and a coverslip after taking the zero timepoint sample. Samples of 10 μL were taken at the stated timepoints and further crosslinking was prevented by the addition of 2 μL of 20 mM Tris/HCl at pH 7.4. The samples were subsequently analyzed by SDS-PAGE followed by immunoblotting for OPA1.

Statistical analysis

Origin software was used for statistical analysis of the data. Values represent mean ± standard error of the mean of independent experiments unless stated otherwise.

Figure Legends

Figure 1. Ablation of Mgm1 can be functionally complemented by a Mgm1-OPA1 hybrid protein.

(A) and (E) Serial dilutions of ΔMGM1 yeast strains expressing the stated human OPA1 variants or hybrid proteins were spotted and grown overnight on synthetic medium without uracil SM GLU-URA and subsequently on medium with a non-fermentable carbon source supplemented with glycerol YPGLY.

(B) Schematic showing the domain structure of chimera 2 (CHIM2) with domains originating from Opa1 or Mmg1 as indicated.

(C) Total cell lysates of WT and ΔMGM1 strains expressing the stated genes were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(E) Representative confocal images (up, brightfield; down, fluorescence) of mitochondrial morphology of the indicated genotypes expressing a mitochondrial targeted GFP.

(F) Morphometric analysis of mitochondrial shape in S. cerevisiae strains of different genotypes. Data represent mean ± standard error of the mean of 3 independent experiment (n=80 cells per experiment)

Table 1. Here-investigated ADOA mutations.

Figure 2. ADOA mutations diminish the ability of the Mgm1-OPA1 hybrid protein to compensate of the absence of Mgm1.

(A) Total cell lysates of WT and ΔMgm1 strains of indicated genotype were separated by SDS-PAGE and immunoblotted with an anti-OPA1 antibody.

(B) Serial dilutions of ΔMGM1 haploid strain coexpressing wild type CHIM2 with a URA3 marker and wild type or mutated CHIM2 with the TRP1 marker and treated with FOA

to lose the URA3 marker. They were grown on minimal SM medium without tryptophan -TRP or uracil URA demonstrating the loss of the wild type protein. Subsequently, they were plated at serial dilutions on glycerol medium YPGLY.

Figure 3. Mutations associated with ADOA plus have a dominant negative effect on wild type CHIM2.

(A) Serial dilutions of ΔMGM1 strain coexpressing wild type CHIM2 and CHIM2 carrying the indicated mutations. To select for strains coexpressing both proteins cells were grown on minimal medium containing uracil and tryptophan SMGLU-URA-TRP and subsequently spotted on glycerol medium YPGLY.

(B) Representative confocal images (right, brightfield; left, fluorescence) of mitochondrial morphology of the indicated genotypes expressing a mitochondrial targeted GFP.

(C) Morphometric analysis of mitochondrial shape in yeast cells coexpressing wild type and mutated CHIM2. Data represent mean ± standard error of the mean of 3 independent experiments (n=80 cells per experiment).

Figure 4. Recombinant Opa1 displays cooperative catalysis and assembles into higher order oligomers.

(A) Schematic showing the domain structure of recombinant Opa1 with indicated origins of either Opa1 protein or the vector backbone (pET).

(B) Representative purification of recombinant OPA1 analyzed by sampling at the indicated steps of the purification process and subsequent separation by SDS-PAGE. Proteins were visualized by immunoblotting with an anti-OPA1 antibody or Coomassie staining. FT = Flowthrough

(C) Kinetics of GTP hydrolysis of 0.2 μg recombinant OPA1 wild type measured by the malachite green assay or reverse phase chromatography (RPC). Data represents mean of 2-3 independent experiments ± standard error of the mean and their respective fitted curve according to the Hill equation.

(D) Crosslinking of oligomers formed by recombinant OPA1 upon exposure to glutaraldehyde vapor. Samples of the indicated timepoints were separated by SDS-PAGE and immunoblotted with an anti-OPA1 antibody. Monomers (M), dimers (D), and tetramers (T) run at the indicated heigths.

Table 2. Kinetic parameters of rOPA1 determined by different GTPase assays

Figure 5. ADOA classic mutations decrease Opa1 cooperative catalysis

(A) Kinetics of GTP hydrolysis of 0.2 µg recombinant OPA1 wild type and the indicated mutant proteins. Data represents mean of 2-3 independent experiments ± the standard errror of the mean and their respective fitted curve according to the Hill equation.

(B) Crosslinking of oligomers formed by the indicated recombinant Opa1 mutants upon exposure to glutaraldehyde vapor. Samples of the indicated timepoints were separated by SDS-PAGE and immunoblotted with an anti-OPA1 antibody. Monomers (M), dimers (D), and tetramers (T) run at the indicated heigths.

(C) Detailed view of (A) emphasizing the sigmoidal curve that is reflected by a Hill coefficient above or below 1.

(D) Densitrometric quantification of oligomeric forms in (B) at 10 minutes of the indicated recombinant mutant Opa1 proteins.

Table 3. Kinetic parameters of rOPA1 and the respective mutant proteins.
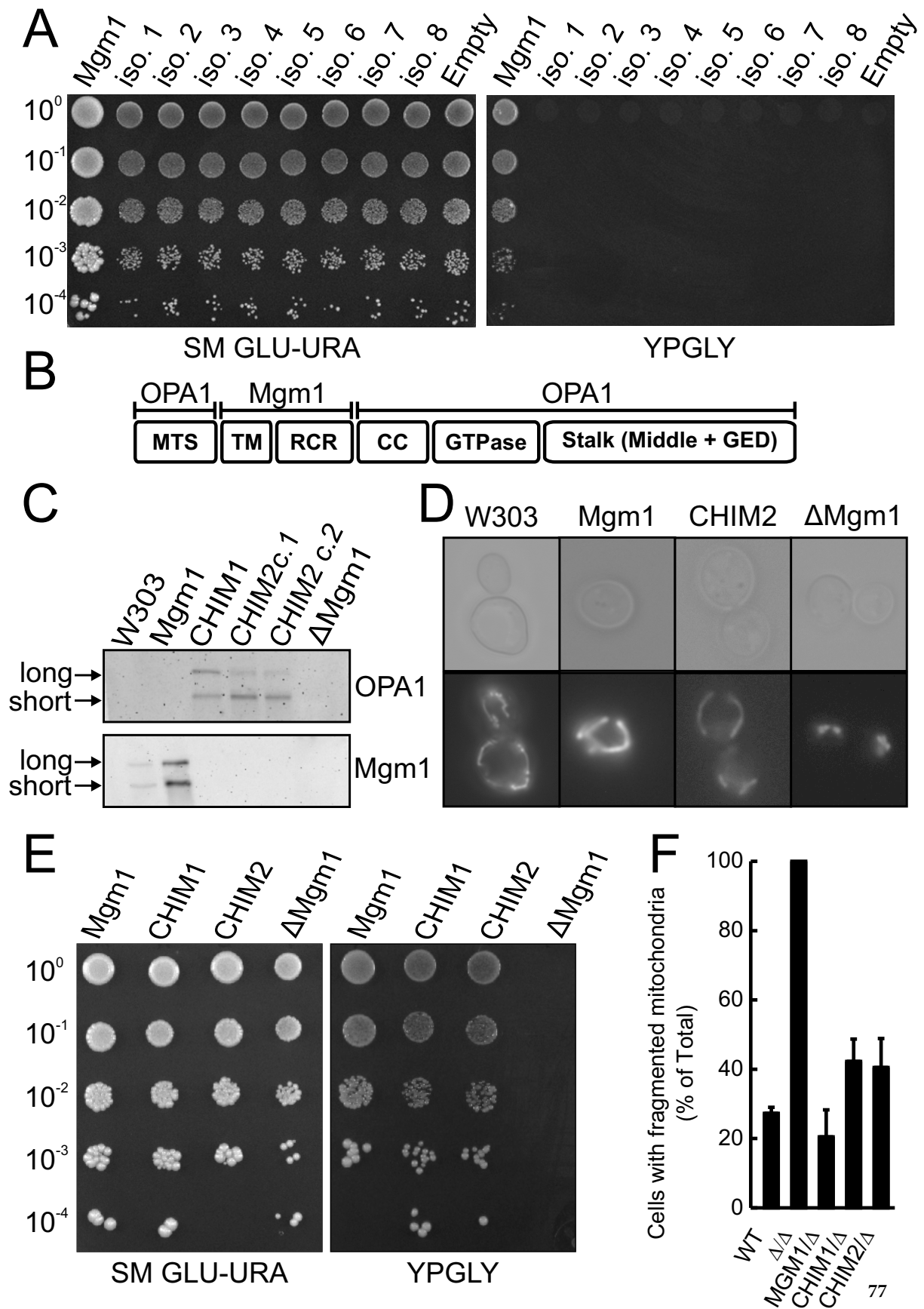
Figure 1

Table 1

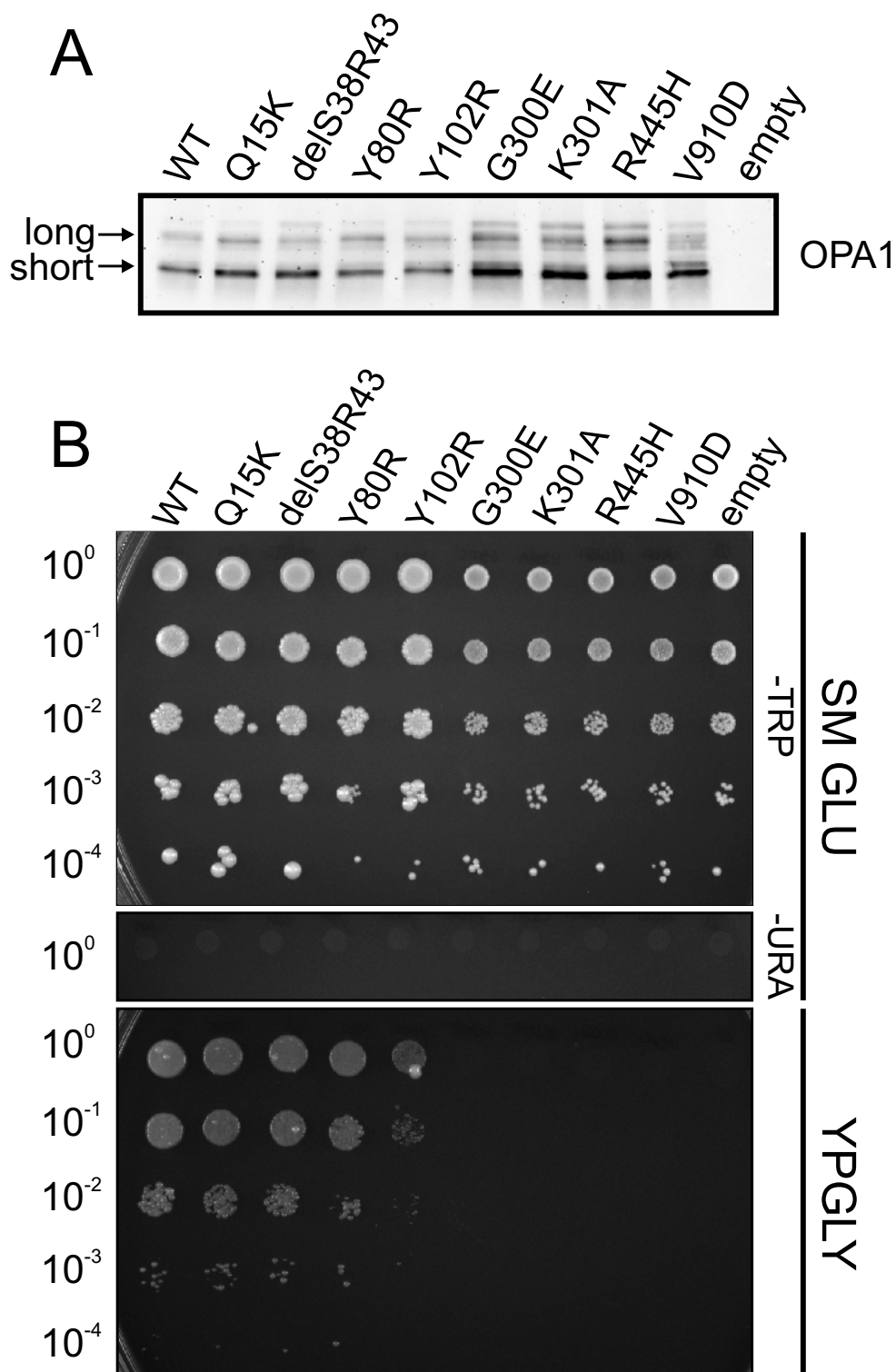|	Domain	Symptoms	Reference
G300E	GTPase	Optic atrophy	Delettre *et al.*, 2000; Olichon *et al.*, 2007
K301A	GTPase	Not ADOA associated	Misaka *et al.*, 2002; Cipolat *et al.*, 2004; Misaka *et al.*, 2006
R445H	GTPase	Optic atrophy, myopathy, deafness, progressive external ophthalmoplegia, ataxia, neuropathy	Amati-Bonneau *et al.*, 2003; Amati-Bonneau *et al.*, 2005; Amati-Bonneau *et al.*, 2008
V910D	GTPase effector	Optic atrophy, myopathy	Amati-Bonneau *et al.*, 2008

Figure 2

Figure 3

Figure 4

A

pET | OPA1 (aa 186 - 960*) | pET

| 22 aa | CC | GTPase | Stalk (Middle + GED) | 12 aa incl. 6xHis |

B

input
FT
imidazole

97
64
51
39
28

coomassie

input
FT
imidazole

OPA1

C

GTP hydrolysis (μM/min)

■ Malachite Green
● RPC

GTP (μM)

D

glutaral
dehyde

time   2.5 5 10 20

460
268

163
117

71

T
D

M
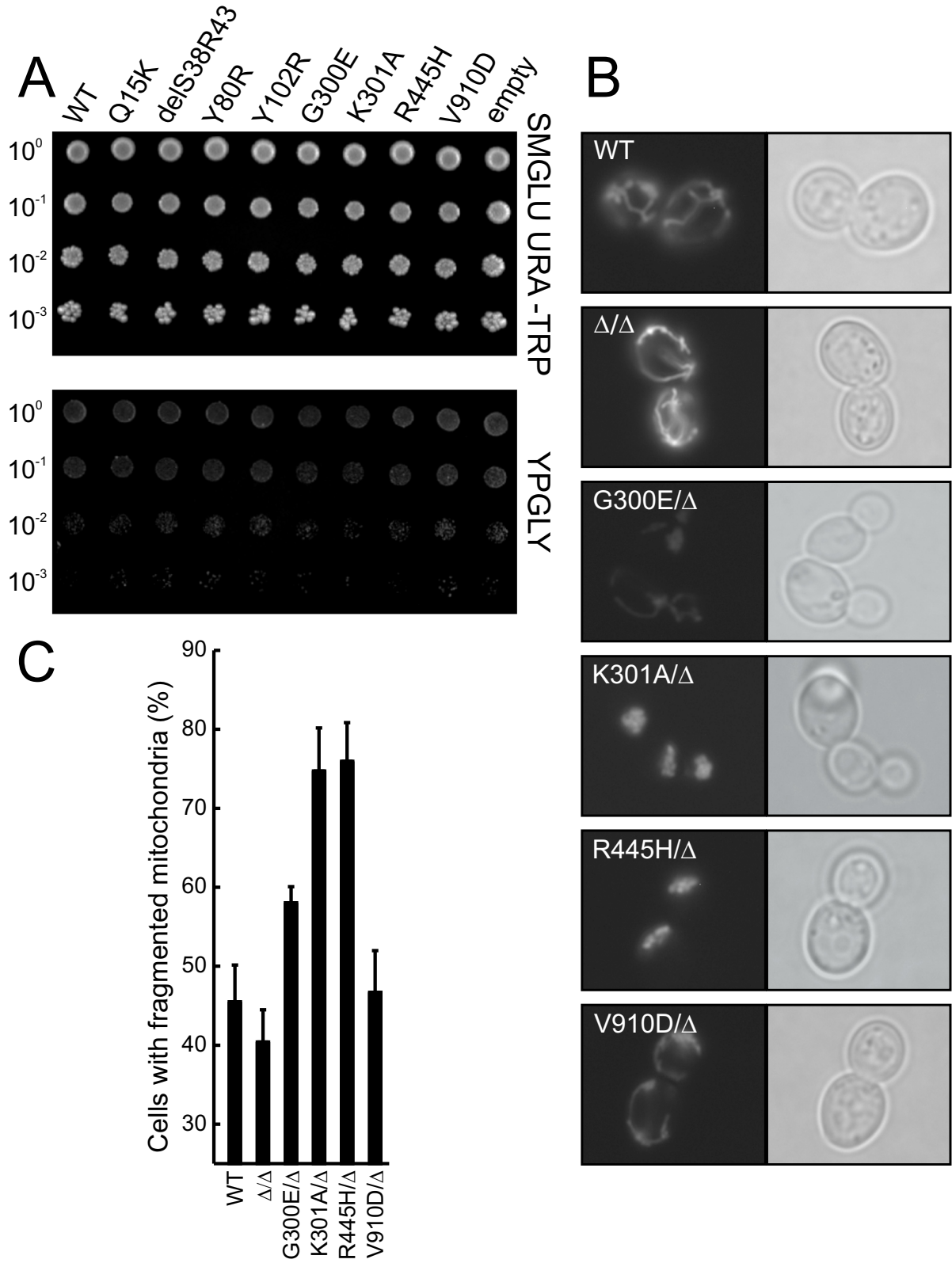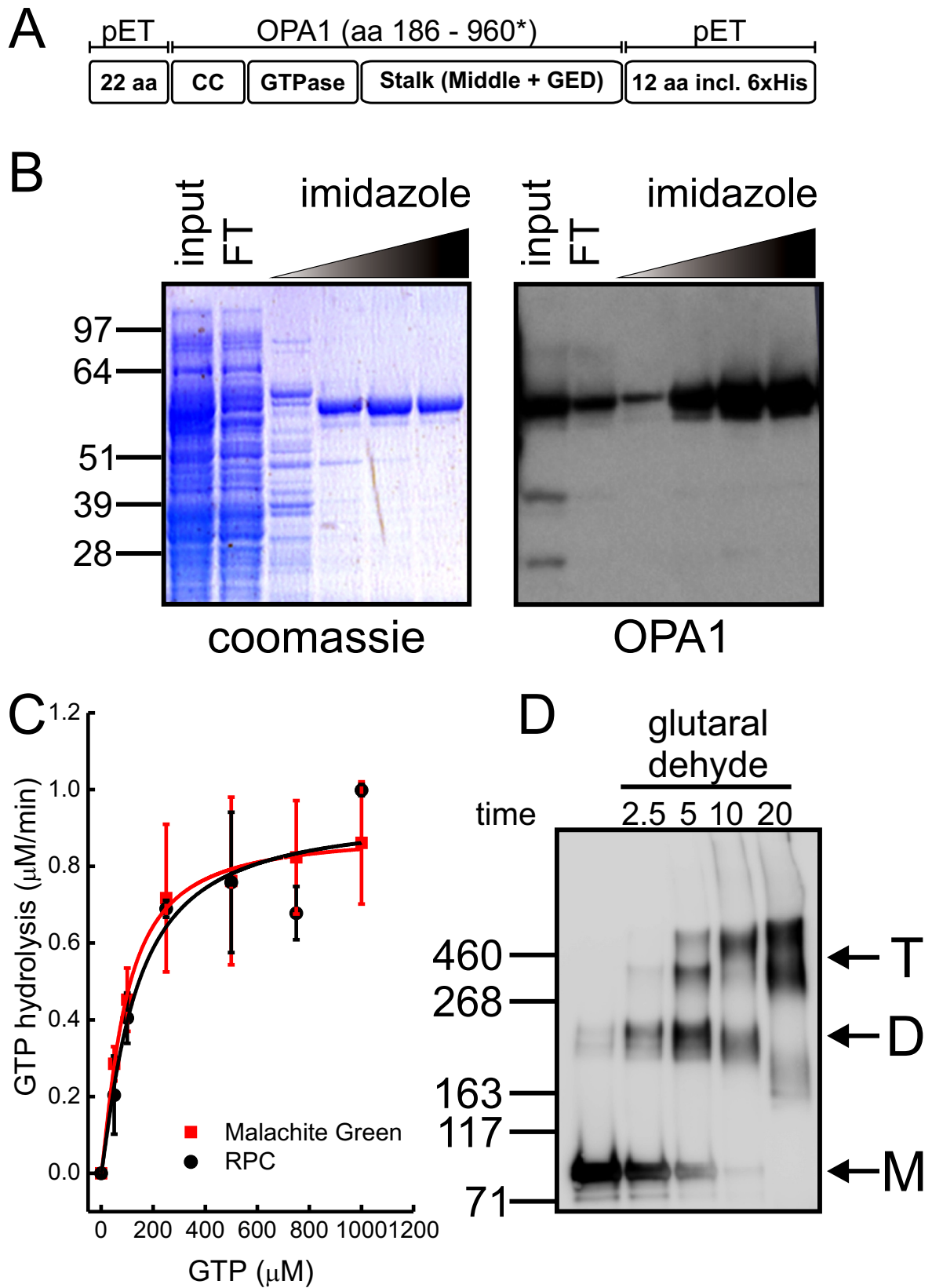
Table 2

	K_m (μM)	k_{cat} (min^{-1})	V_{max} (μM)	Hill coefficient
Malachite Green	106.0 ± 47.9	9.05 ± 1.02	0.89 ± 0.17	1.26 ± 0.82
RPC	156.8 ± 63.7	9.64 ± 1.12	0.94 ± 0.21	1.20 ± 0.68

Figure 5
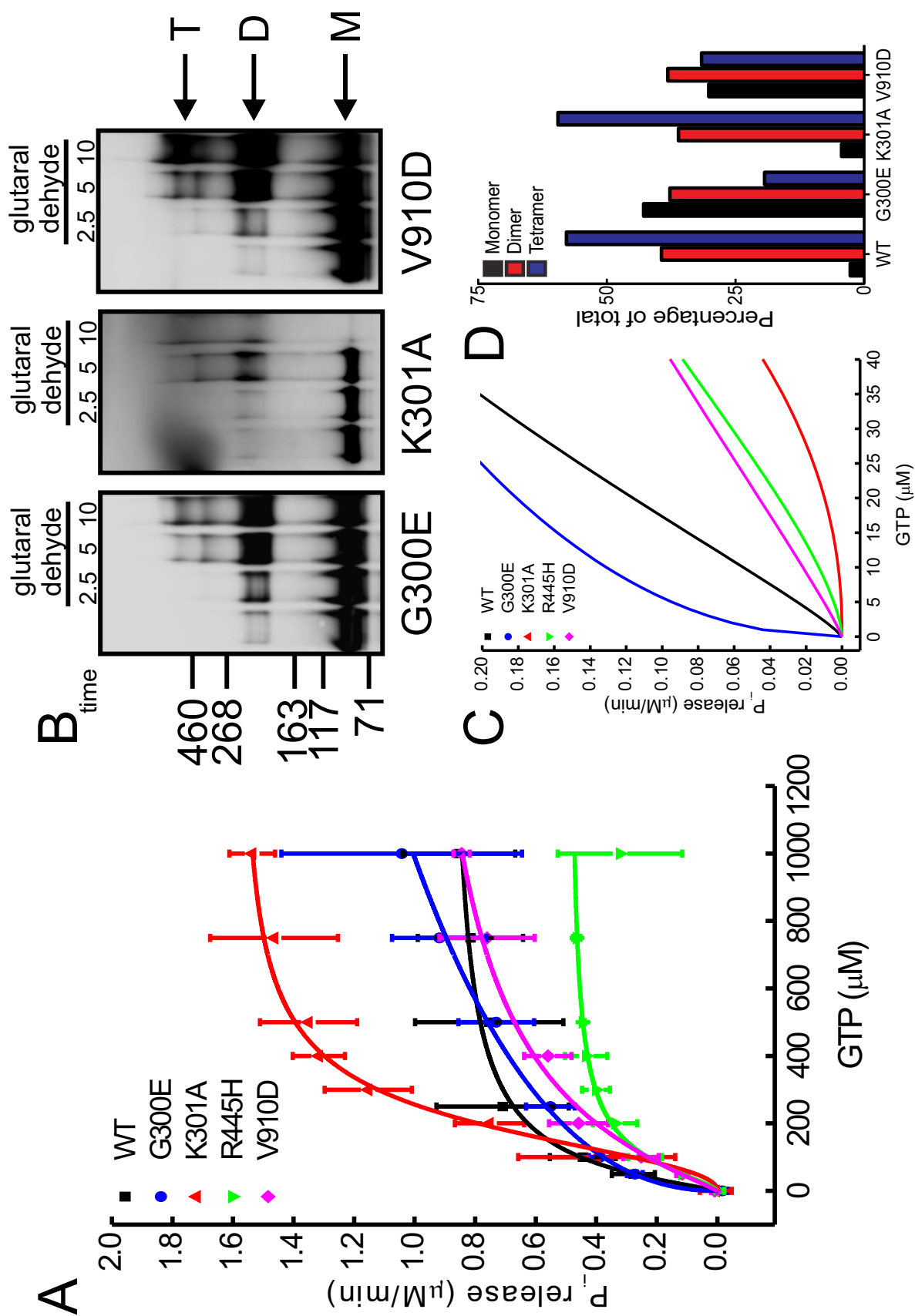
Table 3

	K_m (μM)	k_{cat} (min^{-1})	V_{max} (μM/min)	Hill coefficient
WT	106.0 ± 47.9	9.05 ± 1.02	0.89 ± 0.17	1.26 ± 0.82
G300E	162.8 ± 64.2	10.46 ±1.26	1.21 ± 0.56	0.85 ± 0.52
K301A	270.7 ± 97.84	19.2 ± 2.62	1.81 ± 0.41	1.21 ± 0.47
R445H	146.9 ± 84.91	4.95 ± 0.88	0.47 ± 0.13	1.27 ± 0.85
V910D	311.4 ± 109.2	9.33 ± 1.29	1.01 ± 0.40	0.97 ± 0.41

Supplementary Material

Supplementary Figure S1. Characterization of Mgm1-Opa1 chimera 1.

(A) Schematic showing the domain structure of chimera 1 (CHIM1) with indicated domains originating from Opa1 and Mmg1.

(B) Representative image of mitochondrial morphology of ΔMgm1 yeast cells expressing CHIM1 and a mitochondrial targeted GFP (left, fluorescence; right, brightfield).

(C) Quantification of mitochondrial DNA content represented by COX1 levels. Total DNA content was isolated from the indicated genotypes and COX1 gene levels were determined by real time PCR. Levels were normalized to ACT1 serving as internal control and ratio of the wild type strain was arbitrarily set at 1.
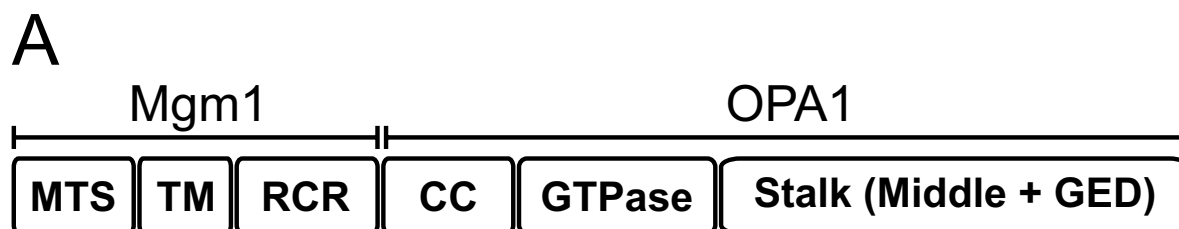
Table S1. Purification pipeline to obtain recombinant Opa1.

Supplementary Figure S2. In vitro hydrolysis requirements of recombinant Opa1.

(A-D) GTP hydrolysis by recombinant Opa1 at the stated conditions at 750 μM GTP stopped after 2.30 hours. Free phosphate content was determined using the malachite green assay.

(E) Timecourse following the hydrolysis of 500 μM GTP by recombinant OPA1 at 37°C in the absence or presence of 500 μM GDP. Reactions were stopped at the indicated timepoints by additions of EDTA and phosphate content was determined using the malachite green assay.
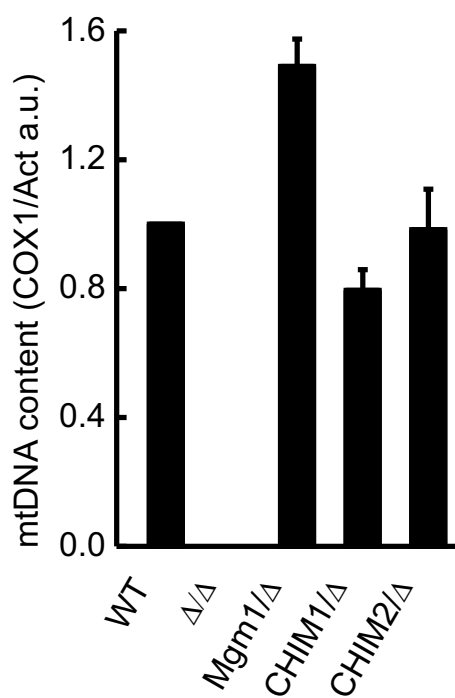
Supplementary Figure S1

A

Mgm1   OPA1

| MTS | TM | RCR | | CC | GTPase | Stalk (Middle + GED) |

B

CHIM1

C

mtDNA content (COX1/Act a.u.)

WT   Δ/Δ   Mgm1/Δ   CHIM1/Δ   CHIM2/Δ
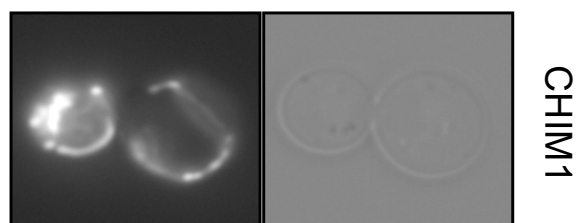
Step	Description	Buffer
Induction	Overnight at 18°C with 0.5 mM IPTG at an optical density of 0.8	Luria Broth (LB)
Lysis	Sonication on ice for 5 times 3 min	Lysis Buffer
	Clearance by centrifugation	40 mM Hepes/KOH, 500 mM NaCl, 10 % glycerol, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, 0.5 % Triton-X-100,, 2 % Tween-20, 1 mM PMSF, 20 mM Imidazole pH 8.0
Purification	Incubation of the supernatant with Ni-NTA beads for 1h at 4°C	Wash Buffer
	Wash rOPA1 bound beads for 30 min with 1 mM ATP and 10 mM MgCl$_2$ in lysis buffer	40 mM Hepes/KOH, 300 mM NaCl, 10 % glycerol, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol, 0.05 % Triton-X-100, 20/40/80 mM Imidazole pH 8.0
	Wash beads in wash buffer with increasing imidazole concentrations (20 mM, 40 mM, 80 mM)	
Elution	Elute rOPA1 with elution buffer with increasing imidazole concentrations (150 mM, 500 mM, 1000 mM)	Elution Buffer
		40 mM Hepes/KOH, 10 % glycerol, 5 mM β-mercaptoethanol, 0.05 % Triton-X-100, 150/500/1000 mM Imidazole pH 7.4
Concentration and buffer exchange	Concentration of pooled elutions by diafiltration and subsequent buffer exchange into storage buffer	Storage Buffer
		40 mM Hepes/KOH, 10 % glycerol, 0.3 mM TCEP, 0.05 % Triton-X-100 pH 7.4

Supplementary Figure S2
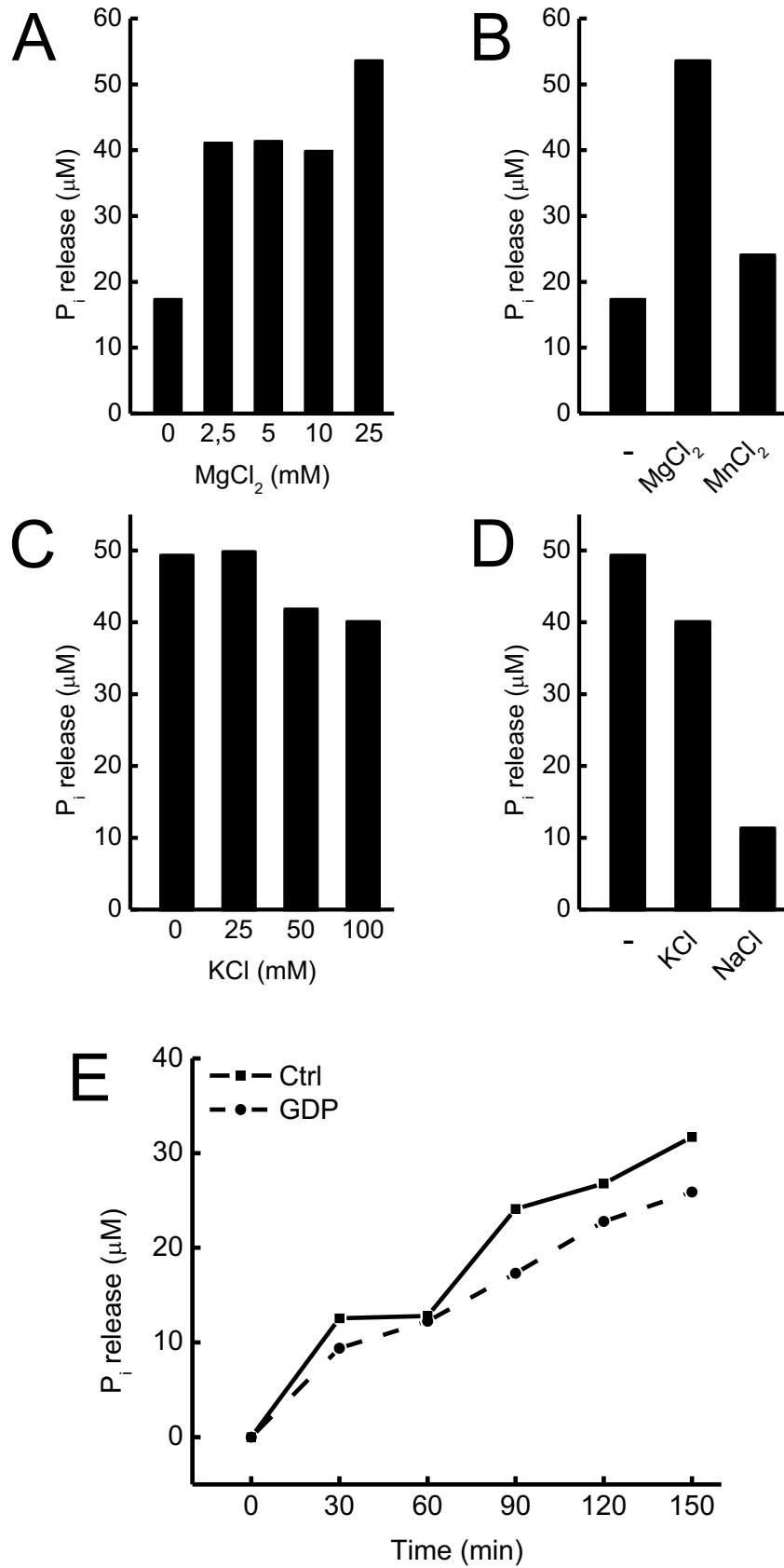
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An *in vitro* high throughput-screening assay identifies small compounds that inhibit the antiapoptotic mitochondrial GTPase OPA1

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Summary

During apoptosis mitochondria undergo cristae remodeling to amplify cytochrome *c* release and activate downstream caspases. This process is controlled by the catalytic activity of the large inner mitochondrial membrane GTPase OPA1, which is upregulated in several tumors stratifying with worse prognosis in non-small cell lung cancer. In order to identify novel lead Opa1 inhibitors, we setup and performed a high-throughput screening that revealed 8 lead compounds that block OPA1 GTPase activity *in vitro*. 10 000 drug-like small compounds were tested for their ability to block GTP hydrolysis of recombinant purified Opa1, identifying moderate and potent inhibitors with different IC_{50}. Our approach identified potential lead compounds for future drug development strategies to produce OPA1 inhibitors that modulate cristae morphology and apoptosis.

Introduction

Mitochondria are crucial organelles in intermediate metabolism, signaling and apoptosis. Programmed cell death shapes development and controls tissue homeostasis in multicellular organisms: not surprisingly its dysregulation is a hallmark of cancer (Thompson, 1995). In mammalian cells, the death receptor pathway and the mitochondrial pathway of apoptosis appear to be linked in certain cell types and culminate in the activation of caspases, cysteine proteases that direct the orderly demise of the cell. Mitochondria participate in the competent activation of caspases by releasing cytochrome c and additional apoptogenic factors from their intermembrane space into the cytosol. Cytochrome *c* in complex with Apaf-1 activates caspase-9 and other downstream caspases (Li et al., 1997). This process is controlled by proteins belonging to the Bcl-2 family (Danial and Korsmeyer, 2004) and is accompanied by mitochondrial morphological and ultrastructural derangement. Ultrastructurally, the mitochondrial inner membrane (IMM) can be further subdivided in an inner boundary membrane and in the cristae compartment, bag-like folds of the IMM connected to it *via* narrow tubular junctions. A set of mitochondria-shaping proteins that impinge on the equilibrium between fusion and fission processes regulates organelle morphology and ultrastructure. In mammalian cells, mitochondrial division is regulated by Dynamin related protein (DRP) 1, mitochondrial fission factor (Mff) and fission (Fis) 1. The Drp1-mediated constriction of the membranes takes place by direct or indirect interaction with its receptors. Mitofusins (MFN) 1 and 2 and Optic Atrophy 1 (OPA1) control mitochondrial fusion in mammals [see (Liesa et al., 2009; Scorrano, 2013) for a review]. Mitofusin are large GTPases of the outer membrane whose deletion impairs mouse embryonic development at different stages. Opa1 is the only dynamin-like GTPase identified so far in the IM: it exists in eight different splice variants, promotes fusion with Mfn1 and controls cristae shape (Cogliati et al., 2013).

Successful approaches to overcome the acquisition of drug resistance by cancer cells are currently lacking. Reduced blood supply to the tumor mass, overexpression of multidrug resistance pumps, of the antiapoptotic members of the Bcl-2 family and of caspase inhibitors that interfere with the pathway of apoptosis exploited by the drugs currently used in the clinics (cis-Pt, doxorubicin, vinblastin for example) all contribute to cancer chemoresistance. In order to circumvent this problem, the concept of combined proapoptotic chemotherapy (Kutuk and Letai, 2008) was put forward and the current approach exploits the use of drugs designed to specifically target the binding interface between Bcl-2 and the proapoptotic members of the family, like ABT-737 (Letai, 2008) whose co-administration of ABT-737 with other inducers of apoptosis can revert chemoresistance of cancer cells. Another key features of cancer cells, their requirement for increased cytoplasmic cytochrome c to induce apoptosis (Zhivotovsky et al., 1998), can be targeted by augmenting cytochrome c release. Complete cytochrome c release requires cristae remodeling, triggered by the BH3 only member of the Bcl-2 family BID (Scorrano et al., 2002), BIK (Germain et al., 2005), BIM, NOXA (Yamaguchi et al., 2008) and BNIP3 (Landes et al., 2010). Opa1 regulates this pathway independently of mitochondrial fusion (Cipolat et al., 2006; Frezza et al., 2006) and using mouse models of Opa1 conditional ablation and overexpression extended the function of Opa1-controlled cristae shape to regulation of cellular proliferation and mitochondrial metabolism (Cogliati et al., 2013).

Genetic ablation or knockdown of OPA1 leads to morphologically aberrant cristae (Cogliati et al., 2013; Frezza et al., 2006; Griparic et al., 2004; Olichon et al., 2003) and increases the sensitivity to apoptotic stimuli due to the facilitated cytochrome c release during apoptotic signaling (Merkwirth et al., 2008; Olichon et al., 2007). Conversely, a mild overexpression increases cristae number and reduces cristae width (Cogliati et al., 2013; Varanita et al., 2015) and hence protects cells from cell death by increasing the resistance to apoptotic stimuli (Frezza et al., 2006). Strikingly, increased OPA1 levels protect mice also *in vivo* from apoptotic damage induced by multiple tissue insults such as Fas-ligand-induced apoptosis (Costa et al., 2010; Varanita et al., 2015). Notably, a handful of studies reported

that Opa1 is overexpressed in several human cancers where high levels of Opa1 correlated with a worst prognosis and an impaired response to therapy (Fang et al., 2012), while its downregulation in cancer cells engages the mitochondrial apoptotic pathway and improves the sensitivity to classical chemotherapeutic agents (Zhao et al., 2013). OPA1 function in apoptosis depends on its GTPase activity and is diminished by mutations in catalytically crucial amino acids in the GTPase domain (Frezza et al., 2006). For this reason we explored the possibility of mimicking the GTPase defective mutant by chemical inhibition of the GTPase activity. To this end we setup and implemented an *in vitro* high-throughput screening assay that is based on the GTPase activity of recombinant OPA1. Screening a library of 10 000 small compounds identified 8 compounds that block GTP hydrolysis by OPA1 which we confirmed by a dose-dependent response. All identified compounds are drug-like compounds as indicated by their physiochemical properties and may be used as leads for the design of potent inhibitors of OPA1 *in vivo*.

Results

Optimization of recombinant OPA1 hydrolysis conditions

In order to identify inhibitors of Opa1, we developed an *in vitro* high-throughput screening to identify drug-like small compounds that inhibit its GTPase activity based on our experience with activity measurements of recombinant Opa1 ref your other paper.

Adapting our assay of Opa1 GTPase activity to a high-throughput mode required appropriate conditions to ensure robustness and stability of the assay readout. We first confirmed that rOPA1 activity can be increasingly inhibited by increasing concentrations by the non-hydrolysable GTP analogues GTPγS and GMPPNP (Figure 1A). We also excluded that 2.5% dimethyl sulfoxide (DMSO), the final concentration of the solvent for the compounds of the library tested in our high throughput screening, affected rOPA1 activity (Figure 1B). Since no inhibitor of OPA1 is so far known we looked for a strong positive control for inhibition. We compared GTPγS inhibitory effect on OPA1 activity to that of ethylenediaminetetraacetic acid (EDTA), which chelates the required Mg^{2+} ions (Figure 1C). While in the GTPγS containing sample a residual activity was measured, EDTA stably and fully inhibited hydrolysis making it a more suitable positive control. Moreover, GTPγS strongly altered the absorbance readout (data not shown), further complicating its use in a high-throughput setting. To understand whether preincubation time affects the inhibition efficiency, we assessed the effect of different GTPγS incubation times on OPA1 activity. Although no significant difference was observed, incubation for 10 minutes appeared to fully inhibit (Figure 1D), in line with the general suggestion of allowing equilibration between enzyme and the potential inhibitor prior to addition of the substrate (Copeland, 2003). These identified parameters were used to design a pipeline for a semi-automated 384-well plates HTS (Table 1).

HTS implementation and hit identification

We performed two rounds of screening a library of 10 000 small compounds at a single concentration ranging from 50 to 200 μM. From this screening we identified 8 small compounds that potentially inhibit OPA1 GTPase activity (Table 2).

High-throughput assays may show large variability with respect to intraplate and interplate results, day-to-day and batch-to-batch comparisons (Goktug et al., 2013). Thus several statistical quality control metrics exist to validate the quality of a developed assay. Certain minimum standards shall be met by the assay design to ensure that positive hits can be distinguished from the noise of the assay. The strictly standardized mean difference SSMD serves to validate the difference between the means and their standard deviation of the positive controls and negative controls (Zhang, 2007). The larger the value of the SSMD between the two controls, the greater the difference between the two populations. In assays like ours, where the positive controls have a value smaller than the negative controls, plates with SSMD values ≤ -3 pass the quality control (Zhang, 2007). In our case the assay remained sufficiently robust throughout the screening as demonstrated by the plate-specific SSMD values (Figure 2A).

Due to the low activity of OPA1 and the assay detection limits the primary screen was performed at a GTP concentration of 500 μM, which is above the Km of OPA1 (K_m = 106 μM). Generally, this enriches the identification of uncompetitive compounds and non-competitive inhibitors instead of competitive inhibitors (Copeland, 2003). Running the assay at higher substrate concentrations was however inevitable in order to obtain a sufficiently robust assay that passed the employed quality metrics. Yet, the conditions were merely met and we compensated for the assay's limitations by setting a conservative threshold that included only the most potent inhibitors of the screen. To identify inhibitors from our primary screen the raw data was normalized as percentage activity relative to the negative controls, which allowed ranking them based on their inhibitory potential (Figure

2B). The majority of the tested compounds showed no effect on OPA1 GTPase activity and hence had a value around 1, similar to the negative controls. Compounds that affected OPA1 activity displayed values smaller than 1 with a minimum of 0, as the positive control values do. Of the compounds having an NPI value between 1 and 0 a compound was regarded as inhibiting when the activity of OPA1 in the respective well was decreased by at least 30% (dashed line in Figure 2B). Compounds that per se reduced the absorbance at the measured wavelength were considered as false positives and excluded from the list (data not shown).

The screened library contained small compounds selected based on their drug-likeness and for their chemical diversity. Their bioavailability was computed based on parameters that evaluate key physiochemical properties that are desirable in small molecules to serve as drug that are described by Lipinski's rule of 5 (Lipinski et al., 2001). Hence, all identified compounds fulfilled the criteria for high drug-likeness, i.e. their molecular weight was below 500 Dalton and their logP was smaller than 5 (Table 2), suggesting good permeability and bioavailability. Only inhibitor #21 is an exception to these rules, yet it is the only compound already in the clinics (Kaur et al., 2002).

Hit validation by concentration response curves

To evaluate the 8 compounds from the primary hit for their ability to inhibit OPA1 GTPase activity we tested them in a dose-response experiment at concentrations ranging from 1 nM to 750 μM (Figure 3). All 8 inhibitors showed a dose response as demonstrated by an increase in inhibition with rising concentrations of the compounds (Figure 3), confirming that they are inhibitors of OPA1 GTPase activity. Fitting a four-parameter curve to the data allowed us to evaluate the potency as half-maximal inhibitory concentration (IC_{50}) and maximal inhibition (I_{max}), as well as the nature of their inhibition, based on the calculation of the Hill coefficient. We grouped the compounds into moderate (Figure 3A to D) and potent inhibitors (Figure 3E to H), according to the maximal OPA1 inhibition they exerted. Compounds that bind a single site on an enzyme yield Hill slopes of 1 and

deviations may indicate that the inhibitor blocks activity nonspecifically. Several of the identified inhibitors show a Hill slope larger than 1 (Figure 3B, 3E, 3F) which may suggest unspecific inhibition through aggregation making future experiments on the solubility of the compounds unavoidable. In conclusion, we identified the first in class *in vitro* inhibitors of OPA1 GTPase activity.

Discussion

Oligomers of OPA1 control cristae width and subsequently cytochrome c release from the intracristal space in a GTPase activity-dependent manner (Frezza et al., 2006). Hence, we reasoned that targeting the GTPase activity of OPA1 will attenuate the pro-apoptotic function of OPA1 and facilitate mitochondria-mediated cell death. Here we describe the development and implementation of a high-throughput compound screen to identify an inhibitor that targets the catalytic domain of OPA1 as approach to induce cristae remodeling.

The HTS assay developed by us identified 8 primary hit compounds that can be developed into lead structures for future drug development. Among these compounds inhibitor #3, and inhibitor #21, namely suramin, has already been focus of previous inhibitor studies.

Historically suramin is a well-known antiparasitic drug used in the clinics to treat African trypanosomiasis. Lately numerous other mammalian targets were identified including G-protein coupled receptors, purinergic P2 receptors, and growth receptors (Kaur et al., 2002). Due to its universal inhibitory effect it served as lead compound in several drug development trials for example as treatment for several cancers such as adrenocortical cancer and prostate cancer (Kaur et al., 2002). The anticancer effect of suramin is ascribed to its ability to block various growth factors and such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Kaur et al., 2002). Moreover, by inhibiting vascular endothelial growth factor (VEGF) activation suramin is thought to exert additional anticancer effect (Waltenberger et al., 1996). The mechanism by which the hexasulfonate compound performs its functions is still not fully understood. In case of G-protein coupled receptors it is thought to uncouple the receptor from the G protein by blocking their interface (Chung and Kermode, 2005) but also an allosteric mechanism of action was observed in case of the serine protease thrombin (Cargnelutti et al., 2012). Moreover, suramin was described to exert mitochondriotoxic effects. It inhibits respiration and glycolysis in trypanosomes (Fairlamb and Bowman, 1980) and similarly, in tumor cells it

inhibits mitochondrial function (Sahdeo et al., 2014) by impairing respiration (Calcaterra et al., 1988; Rago et al., 1991) and inducing depolarization at high concentrations (Eichhorst et al., 2004; Rago et al., 1992). Hence, it will be essential to understand if its mitochondriotoxic properties are related to Opa1 inhibition.

Inhibitor #3 has was identified as hit in a screen to target the HIV fusion protein gp41 (Shibo Jiang, 2004), which was then developed into a lead compound to identify a more potent inhibitor (He et al., 2013). It acts by binding to the hydrophobic pocket of gp41, which inhibits the conformational change that is required for fusiogenic gp41 core to form that then mediates the fusion of HIV-1 with the cell (Shibo Jiang, 2004). Apart from the two aforementioned compounds, none of the remaining 6 was retrieved as active other screenings. This promises high specificity for OPA1 of the here-obtained compounds, which is a desirable property to target a protein in a physiological context.

None of the compounds inhibits OPA1 to 100 % at the tested concentrations. This may reflect partial inhibition or a solubility limitation of the hits (Copeland et al., 2006); concentrations that lie above the solubility limit will not result in an increase in inhibition. In line with this we observed that some of the compounds at high concentrations alter absorbance *per se*, which limits the detection of inhibition at these concentration. Alternatively, partial inhibition is a possible explanation suggesting that the measured activity may derive from more than one enzyme. This may be also be explained by the fact that recombinant OPA1 forms oligomers (Quirin et al., in preparation) resulting in a mixed population of multiple enzyme forms that may differently contribute to the measured GTP hydrolysis. Also endogenous OPA1 is known to form oligomers, which are required for its anti-apoptotic function (Frezza et al., 2006). For this reason, an inhibitor that specifically targets oligomerization or oligomerization-dependent GTPase activity would be particularly promising to modulate OPA1 mediated cristae remodeling.

The steep slope of the IC_{50} curves Hill slopes > 1 of several of the identified compounds may be indicative for a nonspecific inhibition due to the formation of aggregates or cofactor sequestration instead of specifically affecting OPA1 GTPase activity. Enzyme sequestration

by aggregating compounds can however be counteracted by the addition of non-ionic detergents (Acker and Auld, 2014) like Triton-X-100, which is present in our assay conditions. Moreover, an examination of the identified hits for the presence promiscuous compounds (Baell and Holloway, 2010) ruled out their mechanism of action to be aggregation or cofactor sequestration (data not shown). Such promiscuous compounds often result in false positives across a range of assays due to their chemical properties including aggregation-mediated inhibition (McGovern et al., 2002). Hence, more thorough validation of the dose-dependent inhibition of the compounds is required to elucidate their mode of action. An alternative method to measure GTP hydrolysis and an assay that detects direct binding such as surface plasmon resonance (Karlsson, 2004) should clarify these issues.

The here-identified compounds target OPA1 GTPase activity *in vitro*, albeit their potential effect in a physiological environment remains to be tested. *In vitro* high-throughput screenings bear the advantage of measuring specifically the effect on the target of interest circumventing positive hits due to an indirect effect and limitations due to insufficient cell permeability. Moreover, they usually provide a quantitative measurement, as it was the case here, which allows selecting compounds based on their potency (Inglese et al., 2007). Nevertheless, the *in vitro* conditions only poorly mimic the physiological conditions of the targets possibly limiting their impact (Copeland et al., 2006). The purification conditions for OPA1 were optimized to achieve a high protein yield with a reproducible and stable activity. In order to achieve this, we did not account for reproducing the physiological conditions and hence cannot predict the impact the compounds may have *in vivo*. Future experiments on their ability to impact on OPA1, cristae morphology and apoptosis in a cell culture model will provide crucial information on their potency *in vivo*. Moreover, lead compounds for drug development usually show stringent physiochemical parameters (Gleeson et al., 2011). They are more potent compared to primary hits showing typically submicromolar activity, tend to have a more complex buildup and be more flexible and they are generally more lipophilic and less water-soluble (). The compounds identified here inhibit rOPA1 the micromolar range (except for suramin) making them at this point weak lead compounds

for a drug discovery approach. Testing analogues of the compounds will help to determine the important structural features for the inhibition of OPA1 (Hughes et al., 2011) which will help to identify more potent inhibitors that may serve as lead structures. Moreover, targeting drugs specifically to mitochondria by for example conjugating them with a positively charged lipophilic cation that drives accumulation inside the negatively charged mitochondria has been an efficient method to increase the potency of the drugs of interest (Heller et al., 2012).

In conclusion, we identified 8 inhibitors of OPA1 GTPase activity *in vitro* providing a ground for future drug development strategies for *in vivo* modulators of cristae remodeling.

Materials and Methods

Recombinant protein

Recombinant OPA1 was purified as described elsewhere (Quirin et al., in preparation).

GTPase Activity

The enzyme activity of rOPA1 was determined as described elsewhere (Quirin et al., in preparation).

High-throughput GTPase Assay

Recombinant OPA1 was used to screen a commercial small molecule library consisting of 10 000 compounds MyriaScreen, Sigma for interfering small molecules of OPA1-mediated GTP hydrolysis using a 384-well format. GTPase activity was measured using the aforementioned malachite green-ammonium molybdate assay.

The screen was started by plating 0.5 μM of OPA1 and 5 μL of the to be tested compound in 10% DMSO in a total volume of 12 μL per well of a 384 well plate using an automated microplate pipetting system Precision XS, Biotek.

Four columns were used for controls which received 10 % DMSO dissolved in 40 mM Hepes/KOH pH 7.4. Alternating control wells received 5 μL of 0.5 mM EDTA to prevent Mg^{2+}-dependent hydrolysis of GTP serving as positive controls for the inhibition of OPA1 activity. The hydrolysis reaction was initiated after a 10 minute incubation period at room temperature by the addition of GTP mixed with $MgCl_2$ at a final concentrations of 500 μM and 10 mM respectively. The final reaction volume per well was 20 μL. Plates were tape sealed and incubated for 2 hours at 37°C. Subsequently, to stop the hydrolysis reaction 5 μL of 0.5 M EDTA was added per well excluding the ones serving as positive controls. To measure the

contained phosphate content 60 μL of the malachite green-ammonium molybdate mixture was added per well. After 15 minutes incubation at room temperature absorbance was read at 595 nm.

Compound Library

The small molecule library MyriaScreen II consisting of 10 000 compounds Sigma was used for the high-throughput screening. It comprises compounds that fulfill the "Lipinski's rule of 5", indicating their high druglikeness potential (Lipinski et al., 2001). The in DMSO dissolved compounds at 10 mg/mL were diluted 10 times into 40 mM Hepes/KOH pH 7.4 into one sets of 32 384-well per screen repeat and stored at -20°C until use. To reach their final concentration they were further 4 times diluted into the assay plate resulting in final concentrations ranging from 50 to 200 μM.

HTS Data Analysis

The absorbance data obtained from two replicates of the high-throughput screening were analyzed using the Bioconductor/R package, *cellHTS* (Michael Boutros, 2006), modified according to our purposes. The data was normalized in a plate-to-plate fashion using the normalization per inhibition method. The normalized data of each replicate experiment was subsequently standardized to obtain a z-score for each using equation 1 in which y_{kj} is the normalized value for the specific well and M and S are the median and the median absolute deviation of the distribution of sample values across all plates and sample wells of one replicate experiment. Computing their mean summarized the obtained single values per well of the standardized values per replicate.

$$\text{Equation 1} \qquad z_{kj} = \pm (y_{ki} - M)/S$$

To identify positive modulators of OPA1 GTPase activity a threshold was set considering compounds that reduce the activity by at least 30%. Of the resulting compounds only compounds that appeared in the data determination of the replicates as individual data sets and the replicate screen as data set were identified as positive hits. Outliers with values lower than the average of the positive control wells were excluded from the analysis. The strictly standardized mean difference SSMD per plate was determined according to Zhang et al. 2007.

IC$_{50}$ determination

GTPase activity was measured using the aforementioned malachite green-ammonium molybdate assay. OPA1 was incubated with the compounds at concentrations 1 nM to 750 µM for 10 minutes at room temperature. Subsequently, GTP hydrolysis was initiated by adding GTP and MgCl$_2$ to reach final concentrations of 500 µM and 10 mM respectively. The hydrolysis was aborted after 2 hours at 37°C by the addition of 5 µL of 0.5 mM EDTA per well. The contained phosphate concentrations were measured as described above. Values are expressed as normalized percent inhibition considering wells containing EDTA at timepoint zero as positive controls and wells containing a vehicle control as negative control. To obtain a half maximal inhibitory concentration IC$_{50}$ based on the dose-response curves are fit with a four parameter logistic fit using OriginPro software.

Figure Legends

Figure 1. Optimization of OPA1 activity for HTS settings.

(A) GTP hydrolysis by recombinant OPA1 at 750 μM GTP in the presence of GTPγS or GMPPNP at the stated concentrations. Reactions were stopped after 90 minutes by addition of EDTA phosphate content was measured using the malachite green assay. Data is expressed as percentage of the negative control that lacked GTP analogues.

(B) Timecourse following the hydrolysis of 500 μM GTP by recombinant OPA1 at 37°C in the absence or presence 2.5% DMSO. Reactions were stopped at the indicated timepoints by additions of EDTA and phosphate content was determined using the malachite green assay.

(C) Timecourse following the hydrolysis of 500 μM GTP by recombinant OPA1 at 37°C in the absence or presence 2.5% DMSO. Reactions were stopped at the indicated timepoints by additions of EDTA and phosphate content was determined using the malachite green assay.

(D) Hydrolysis of 750 μM GTP by recombinant OPA1 in the presence of 1000 μM GTPγS. GTPγS was added to OPA1 and incubated for the indicated time periods prior to initiating GTP hydrolysis. Reactions were stopped after 60 minutes by additions of EDTA. Data is expressed as percentage of control wells that lack GTPγS.

Table 1. High-throughput screening steps including descriptions on the individual components.

Figure 2. High-throughput evaluation and data overview.

(A) Strictly standardized mean difference values per plate plotted against the plate number of the 32 tested plates. The dashed lines at a y-value of -3 highlights the threshold for a robust assay. Values represent the average per plate of two replicates of the HTS.

(B) Average normalized percent inhibition (NPI) values per well of the two replicate experiments were plotted against their z-score. NPI values of 1 represent zero inhibition while values of 0 represent values of 100 % inhibition computed based on the positive control wells. The dashed line indicates the threshold set at 0.7 corresponding to 30% inhibition of the measured GTPase activity of OPA1.

Table 2. Active compounds identified in the primary screen that block OPA1 GTPase activity.

NPI, Normalized percent inhibition

MW, Molecular weight

Figure 3. Dose response curves for the individual active compounds of the primary screen.

(A-H) Normalized percent inhibition values determined based on OPA1 GTPase activity plotted against the stated logX of increasing compound concentrations that ranged from 1 nM to 750 μM. Raw data was normalized using EDTA containing wells as positive controls and wells with a vehicle control as negative controls.

A four parametric curve was fit to the data to obtain the stated half-maximal inhibitin value IC_{50}), maximum inhibition I_{50} and Hill slope values using OriginPro software. Data represents mean of 3 independent experiments ± standard error of the mean.

(A-H) Moderate inhibitors of OPA1 GTPase activity.
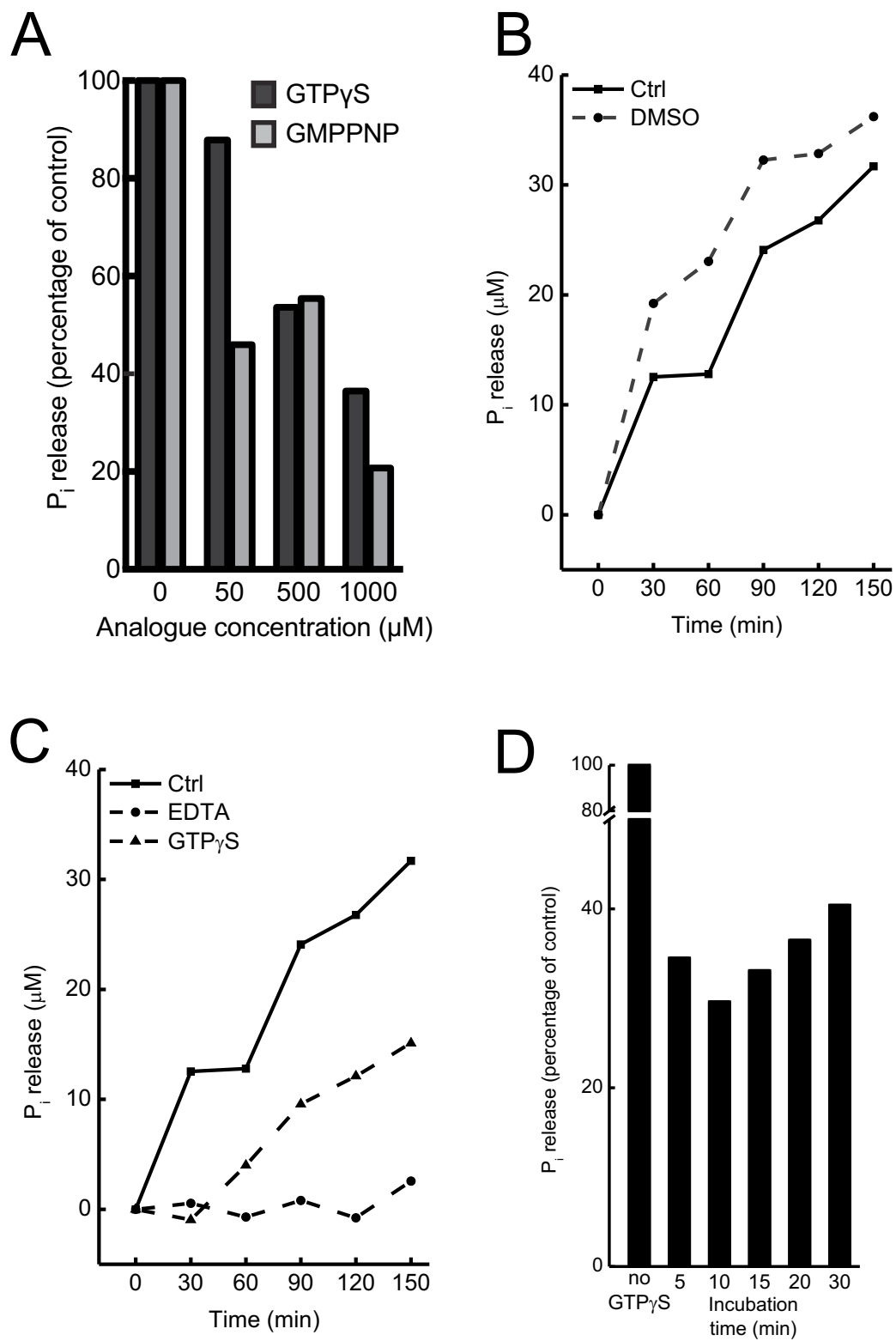
(E-H) Potent inhibitors of OPA1 GTPase activity.

Figure 1

Table 1

Step	Parameter	Value	Description
1	Transfer compounds	5 µL	From 200 – 830 µM in 10% DMSO
2	Plate recombinant Opa1	8 µL	From a 1.2 µM stock in storage buffer
3	Incubation time	*10 min*	At room temperature
4	Add EDTA to positive control wells	5 µL	From a stock at 0.5 M, pH 8.0
5	Plate GTP into all wells	7 µL	From a stock containing 1.4 mM GTP and 28.6 mM $MgCl_2$
	Assay volume / well = 20 µL		*Containing 0.5 µM rOpa1, 500 µM, 10 mM $MgCl_2$, 2.5 % DMSO*
6	Incubation time	*2 hours*	Sealed at 37°C
7	Add EDTA to all wells excluding positive control wells	5 µL	From a stock at 0.5 M, pH 8.0
8	Add malachite green-ammonium molybdate mix	60 µL	From a stock with 1.5 mM malachite green and 8 mM ammonium molybdate
9	Incubation time	*15 min*	At room temperature
10	Assay readout	*595 nm*	

Figure 2
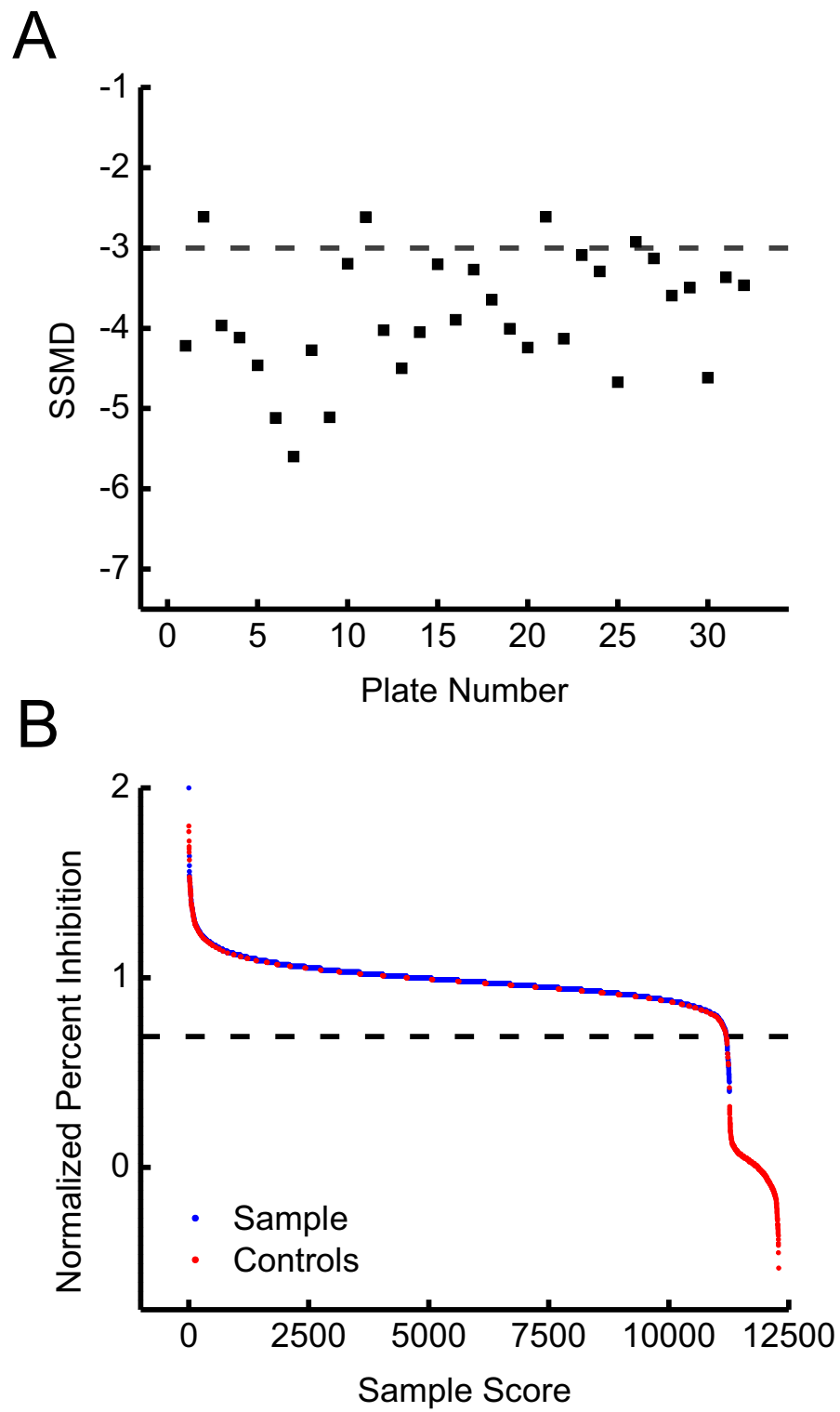
Table 2

	Formula	MW (Dalton)	NPI value of HTS	LogP
#2	$C_{14}H_{10}N_2O_5S$	318.31	56	3.49
#3	$C_{13}H_{13}NO_3$	231.25	53	3.98
#5	$C_{12}H_6Cl_2N_2O_6S$	377.16	62	4.13
#9	$C_9H_6N_2O_3S$	222.22	46	1.80
#10	$C_5H_4N_4S_3$	216.31	48	-0.82
#14	$C_{14}H_7N_3O_4$	281.23	55	1.89
#16	$C_{15}H_9NO_6S$	331.31	55	1.87
#21	$C_{51}H_{40}N_6O_{23}S_6$	1297.28	40	5.58

Figure 3

A — Inhibitor #2
IC_{50} = 106.26
Hill slope = 0.94
I_{max} = 0.43

B — Inhibitor #3
IC_{50} = 101.72
Hill slope = 24.2
I_{max} = 0.18

C — Inhibitor #5
IC_{50} = 10.92
Hill slope = 1.13
I_{max} = 0.28

D — Inhibitor #14
IC_{50} = 4.38
Hill slope = 1.21
I_{max} = 0.44

E — Inhibitor #9
IC_{50} = 305.04
Hill slope = 2.37
I_{max} = 0.68

F — Inhibitor #10
IC_{50} = 177.52
Hill slope = 1.72
I_{max} = 0.62

G — Inhibitor #16
IC_{50} = 363,03
Hill slope = 0.59
I_{max} = 0.98

H — Inhibitor #21
IC_{50} = 0.35
Hill slope = 1.05
I_{max} = 0.47

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Conclusion and Future Directions

The work presented in this thesis contributes to the understanding of the OPA1 and identifies tools that can be used to deepen our knowledge of the physiological roles OPA1 impacts on.

In the first part of the thesis, we established a yeast model to test ADOA-associated mutations in OPA1 that can be used for future experiments to screen the impact of different mutations and analyzed how ADOA classic- and plus-associated mutations impact on OPA1 function *in vitro* as well as *in vivo*. We find that all analyzed ADOA-associated mutations impair OPA1 GTPase-dependent functions. Moreover, while ADOA classic mutations result in a loss of function phenotype, ADOA plus mutations have a dominant negative effect on the wild type protein. Based on our *in vitro* data we suggest that the difference between the two groups of mutations resides in the impact they have on the cooperative GTPase activity of OPA1. We conclude that ADOA classic and ADOA plus associated mutations can be grouped *in vivo* and *in vitro* according to their impact on the cooperative GTPase activity of OPA1.

Other groups have proposed similar mechanisms to be the cause for differently severe forms of ADOA. We further our knowledge in this field however by linking the cooperative GTPase activity to these two different phenotypes. We show here, that OPA1, like other members of the dynamin-like family shows cooperative GTPase activity and oligomerizes *in vitro*. We propose that the cooperativity derives from hydrolysis-dependent oligomerization, which will be elucidated in future structural analyses. Moreover, a resolved protein structure could indicate the impact of ADOA-mutations on OPA1 and its oligomerization states. Understanding the molecular mechanisms giving rise to ADOA will enable therapy development.

In the second part of the thesis we focused on chemically targeting the catalytic domain

of OPA1. The here-described high-throughput screening led to the identification of 8 inhibitors of OPA1 *in vitro*. Their physiochemical properties suggest that they are permeable and soluble compounds implying high bioavailability.

Further investigations will be needed to understand the importance of OPA1 GTPase activity for its diverse functions, but we hypothesize that blocking OPA1 GTPase activity will facilitate intrinsic cell death and thus these inhibitors may represent a promising anti-cancer therapy when co-administered with potent chemotherapeutic drugs that induce cell death.

The quantitative approach utilized in this high-throughput screening allows identifying compounds that positively modulated OPA1-GTPase activity. A positive modulator of OPA1 could also be considered in the treatment of ADOA patients in which haploinsufficiency is the likely pathomechanism.

In conclusion, this work reveals underlying molecular mechanisms that may determine the clinical outcome of ADOA mutations. Their impact on the cooperative GTPase activity of OPA1 is a major feature of ADOA plus mutations potentially explaining the dominant negative phenotype observed compared to ADOA classic mutations that merely reduce OPA1 GTPase activity.

Moreover, we discuss the identification of small molecule inhibitors using an high-throughput screening for inhibitors of the catalytic domain of OPA1. These inhibitors may be used as the basis for lead compounds to design potent small molecule inhibitors of OPA1 to induce mitochondrial cristae remodeling.

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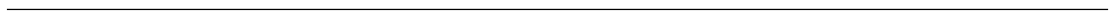
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