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CHANGES IN MITOCHONDRIAL *CRISTAE* STRUCTURE DETERMINE CELL VIABILITY

IN MODELS OF HUNTINGTON'S DISEASE

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1 Riassunto dell'attività svolta

I mitocondri sono organelli esseziali nella vita della cellula: essi rappresentano la maggiore fonte di ATP (Danial et al., 2003) e regolano importanti vie di trasduzione del segnale, dall'omeostasi del Ca^{2+} (Rizzuto et al., 2000) all'apoptosi.

In seguito ad uno stimolo apoptotico i mitocondri rilasciano citocromo *c* e altre proteine solubili nello spazio intermembrana che sono necessarie, nel citoplasma, per l'attivazione e l'amplificazione della cascata del segnale che porta al disassemblamento della cellula. La maggior parte del citocromo *c* e dei componenti della fosforilazione ossidativa sono localizzati all'interno delle *cristae*. Grazie ai notevoli sviluppi della microscopia elettronica associata a ricostruzione tridimensionale il modello di ultrastruttura dei mitocondri è stato recentemente rivoluzionato; le *cristae*, dapprima identificate come semplici invaginazioni della membrana mitocondriale interna (IMM) (Palade, 1952), risultano essere compartimenti distinti di IMM, separati dallo spazio intermembrana da giunzioni tubulari strette definite *cristae junctions* (Mannella et al., 1994). Durante l'apoptosi, per garantire un completo rilascio di citocromo *c* le singole *cristae* si fondono tra loro e le giunzioni tubulari strette si allargano: questo processo, noto con il nome di *cristae remodelling*, è associato alla mobilizzazione del citocromo *c* dal compartimento intracristale allo spazio intermembrana, evento necessario per il suo successivo rilascio attraverso la membrana mitocondriale esterna (Scorrano et al., 2002). Il rimodellamento ultrastrutturale è accompagnato da una massiva frammentazione del reticolo mitocondriale.

La forma del reticolo mitocondriale è determinata dall'equilibrio tra eventi di fusione e fissione, controllati da numerose proteine tra cui le GTPasi simili a dinamine. In cellule di mammifero, la fusione mitocondriale è controllata dalle proteine mitofusina-1 (MFN1) e mitofusina-2 (MFN2) nella membrana esterna e da OPA1 nella membrana mitocondriale interna (Olichon et al., 2002). Nel nostro laboratorio è stato dimostrato che OPA1 promuove la fusione dei mitocondri solo in presenza di mitofusina 1 (Cipolat et al., 2004). La fissione richiede il passaggio aggiuntivo della traslocazione della proteina Drp1 dal citosol ai mitocondri, dove si ancora a hFis1, il suo adattatore molecolare nella OMM. L'oligomerizzazione di Drp1 fornisce la forza meccanica per costringere le membrane mitocondriali fino alla frammentazione dell'organello (Hinshaw et al., 1999; Smirnova et al., 2001). La traslocazione di Drp1 ai mitocondri dipende dalla

sua defosforilazione ad opera della fosfatasi calcineurina e di altre fosfatasi (Cereghetti et al., 2008).

Un crescente numero di evidenze sperimentali suggerisce che le proteine che regolano la morfologia dei mitocondri giochino un ruolo nella morte cellulare. Nel nostro laboratorio è stato recentemente dimostrato che OPA1 protegge dall'apoptosi prevenendo il rilascio di citocromo *c* in maniera indipendente dal suo ruolo profusogeno. OPA1 agisce controllando il processo di rimodellamento delle *cristae* e mantiene strette le giunzioni delle *cristae* attraverso la formazione di oligomeri, costituiti da una forma solubile localizzata nello spazio intermembrana e una forma integrale della IMM (Frezza et al., 2006; Cipolat et al., 2006). Inoltre, numerosi lavori hanno dimostrato che l'espressione di un mutante dominante negativo di Drp1 è in grado di prevenire la frammentazione mitocondriale, il rilascio di citocromo *c* e la morte cellulare indotti da stimoli apoptotici intrinseci (Frank et al., 2001). È' interessante notare che Drp1 sembra regolare in modo specifico il rilascio di citocromo *c* suggerendo una sua capacità di regolare la sub compartimentalizzazione mitocondriale di questo fattore pro-apoptotico (Parone et al., 2006).

Lo scopo della mia tesi è stato quello di studiare il ruolo della frammentazione mitocondriale regolata da calcineurina nella morte cellulare e nella patogenesi della Corea di Huntington.

Recentemente, nel nostro laboratorio è stato dimostrato che la depolarizzazione mitocondriale causa l'attivazione della fosfatasi citosolica calcineurina. Calcineurina defosforila Drp1 in corrispondenza del residuo di serina 637, stimolandone la traslocazione ai mitocondri e causando la frammentazione degli organelli (Cereghetti et al., 2008). Allo scopo di caratterizzare l'importanza di questa via del segnale nell'apoptosi abbiamo focalizzato la nostra attenzione su un nuovo specifico inibitore di calcineurina: il peptide PPD1, che corrisponde al dominio peptidilprolil isomerasico della immunofillina FKBP52. PPD1 è codificato geneticamente e quindi risulta essere più stabile degli inibitori farmacologici. Attraverso esperimenti di immunoprecipitazione, abbiamo dimostrato che PPD1 non influenza l'interazione di Drp1 con il complesso motore contenente la dineina, anch'esso coinvolto nella traslocazione di Drp1 ai mitocondri. Un'analisi cinetica tramite microscopia confocale di cellule esprimenti una variante spettrale della *green fluorescent protein*, la *red fluorescent protein* (mtRFP), specificatamente diretta alla matrice mitocondriale, ci ha permesso di verificare che l'espressione di PPD1 inibisce fortemente la frammentazione del reticolo indotta da

depolarizzazione mitocondriale. Questo effetto può essere attribuito alla riduzione di traslocazione di Drp1 ai mitocondri. Abbiamo poi voluto controllare se l'inibizione della frammentazione esercitata da PPD1 potesse interferire con l'apoptosi. A questo scopo abbiamo analizzato la cinetica della morte cellulare indotta da stimoli apoptotici intrinseci. L'analisi ha rivelato che l'espressione di PPD1 è in grado di proteggere dalla morte cellulare indotta da staurosporina, perossido di idrogeno, etoposide e tapsigargina. Inoltre, un saggio clonogenico ha confermato l'effetto protettivo di PPD1, poiché cellule esprimenti il peptide hanno dimostrato un'aumentata capacità di formare colonie dopo il trattamento con staurosporina. Per capire se PPD1 potesse influenzare il rilascio di citocromo *c* abbiamo espresso la proteina mtRFP come marker mitocondriale e abbiamo immunodecorato il citocromo *c* con un anticorpo coniugato ad un fluoroforo verde (FITC). L'overespressione di PPD1 è in grado di prevenire il rilascio di citocromo *c* in risposta alla staurosporina. Abbiamo poi voluto accertare se l'inibizione dell'apoptosi da parte di PPD1 dipendesse in modo specifico dalla sua azione sulla proteina Drp1. A questo scopo è stato necessario escludere che l'effetto di PPD1 fosse dovuto all'inibizione della fosforilazione di altri substrati di calcineurina coinvolti nell'apoptosi, come il membro della famiglia di proteine Bcl2 BAD. Ci siamo quindi avvalsi di un approccio genetico andando ad analizzare l'effetto di PPD1 sulla morte cellulare in fibroblasti murini embrionali wt e ko per BAD. I nostri esperimenti hanno dimostrato che BAD non è necessaria per l'effetto anitapoptotico di PPD1, poiché il peptide protegge dalla morte cellulare anche cellule BAD ko. Inoltre, la co-espressione di PPD1 e un mutante dominante negativo di DRP1 (DRP1^{K38A}) non risulta in un effetto di protezione aggiuntivo, indicando che le due proteine agiscono nella stessa via di trasduzione del segnale. Questi dati rappresentano un'ulteriore conferma del ruolo decisivo giocato dalla frammentazione mitocondriale e dai modulatori della morfologia mitocondriale nell'apoptosi (Cereghetti, Costa et al., in revisione, Cell Death and Differentiation).

Un crescente numero di studi dimostra che in condizioni patologiche caratterizzate da aumentata apoptosi, come nelle patologie neurodegenerative, i mitocondri presentano alterazioni della morfologia del reticolo e della loro ultrastruttura. In particolare, analisi di microscopia elettronica di linfoblasti da pazienti affetti da Corea di Huntington hanno evidenziato mitocondri con profonde alterazioni ultrastrutturali comparabili a quelle osservate durante il processo di rimodellamento del *cristae*. La Corea di Huntington è una patologia neurodegenerativa genetica causata dalla anomala espansione di un

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dominio poliglutamminico nella proteina *Huntingtin*. La principale caratteristica clinica della patologia è la perdita selettiva di neuroni GABA-ergici dello *striatum* che causa l'insorgenza di disturbi motori e cognitivi. Abbiamo deciso di caratterizzare le alterazioni morfologiche dei mitocondri e il loro effetto sulla morte cellulare in due diversi modelli della malattia: linfoblasti immortalizzati isolati da pazienti e precursori di neuroni striatali derivati da un modello murino transgenico avente una espansione delle ripetizioni CAG nel gene endogeno per la proteina *huntingtin*. L'analisi tramite microscopia confocale di cellule trasfettate con una proteina fluorescente gialla diretta ai mitocondri (mtYFP) ha rivelato che le cellule aventi la mutazione presentavano un reticolo mitocondriale frammentato se paragonate alle relative cellule di controllo. È importante notare che la frammentazione è risultata essere più evidente in linfoblasti da pazienti omozigoti che da eterozigoti indicando una correlazione tra la severità del fenotipo e il genotipo. Abbiamo poi controllato se il fenotipo morfologico potesse essere causato da alterazioni nei livelli delle proteine che controllano la forma dei mitocondri. I nostri esperimenti non hanno rivelato differenze nei livelli di espressione, ma abbiamo osservato che la proteina Drp1 era maggiormente associata ai mitocondri isolati da cellule di Huntington che dalle wt. Inoltre, esperimenti di crosslinking hanno evidenziato una aumentata oligomerizzazione di Drp1 nelle cellule aventi la mutazione. Questi dati hanno suggerito che l'eccessiva attivazione dell'apparato di fissione potesse essere la causa della frammentazione osservata. Abbiamo inoltre riscontrato alterazioni nella abbondanza relativa delle isoforme di OPA1 allo stato stazionario e del loro processamento in risposta a trattamento con staurosporina. Questo dato spinge ad ipotizzare un potenziale meccanismo molecolare attraverso il quale Drp1 possa regolare la struttura delle *cristae*. Abbiamo poi cercato di correggere il fenotipo morfologico promuovendo la fusione o inibendo la fissione dei mitocondri. L'overespressione di proteine profusogene OPA1 e MFN1 e di mutanti dominanti negativi di Drp1 e di calcineurina è risultata nella correzione del difetto morfologico. Il passo successivo è stato quello di indagare il significato funzionale delle alterazioni osservate, in particolare nella morte cellulare. Analisi tramite citofluorimetria condotta sui linfoblasti ha evidenziato una aumentata sensibilità alla morte indotta da staurosporina in cellule da paziente. Il risultato è stato confermato nel modello neuronale murino in cui è stato analizzato il processamento della proteina PARP (poly ADP-ribose polymerase), un evento caratteristico dell'apoptosi. L'overespressione di OPA1 e di DRP1^{K38A} o il trattamento con l'inibitore farmacologico di calcineurina FK506 ha riportato la sensibilità all'apoptosi in cellule di

Huntington a livelli paragonabili a quelli delle cellule di controllo. Al contrario, l'overespressione di MFN1 non ha conferito protezione contro la morte cellulare, suggerendo che la fusione mitocondriale *per se* non è sufficiente a contrastare gli stimoli apoptotici. L'analisi della cinetica del rilascio di citocromo *c* da mitocondri isolati in risposta al trattamento con lo stimolo apoptotico cBID, attraverso l'uso di uno specifico saggio ELISA, ha evidenziato che mitocondri da cellule di Huntington rilasciano il citocromo *c* più velocemente che i relativi controlli. Inoltre, esperimenti di crosslinking hanno dimostrato che l'aumentato rilascio di citocromo *c* correla con una più rapida distruzione degli oligomeri di OPA1. L'aumentato rimodellamento delle *cristae* nei mitocondri da cellule aventi l'espansione poliglutamminica è stato confermato attraverso analisi di microscopia elettronica in seguito a trattamento con staurosporina. In cellule mutate il numero medio di *cristae* per mitocondrio allo stato stazionario è risultato ridotto e le *cristae* risultano profondamente alterate e riorganizzate dopo trattamento con lo stimolo apoptotico. L'overespressione di OPA1 e di DRP1^{K38A} ha aumentato il numero di *cristae* correttamente strutturate e ha inibito il loro rimodellamento durante l'apoptosi.

In conclusione, i dati presentati in questa tesi di dottorato mostrano che la regolazione della frammentazione mitocondriale da parte dell'asse calcineurina-DRP1 gioca un ruolo cruciale nella progressione della cascata apoptotica. Abbiamo potuto dimostrare che questa via è alterata nella Corea di Huntington e partecipa all'aumentata apoptosi che caratterizza questa patologia neurodegenerativa. Inoltre, questo modello ci ha permesso di chiarire la relazione tra la regolazione della forma del reticolo mitocondriale e la struttura delle *cristae*, identificando in OPA1 un possibile nesso molecolare (Costa et al., sottomesso, EMBO Mol. Med.).

2 Summary

Mitochondria are essential organelles for the life of the cells since they are the major source of cellular ATP (Danial et al., 2003), they regulate Ca^{2+} signalling (Rizzuto et al., 2000) and programmed cell death (Green and Kroemer, 2004). Mitochondria amplify the intrinsic apoptotic pathway by releasing protein cofactors, like cytochrome *c*, that are crucial for the activation of effector caspases required for cell demise. The majority of cytochrome *c* and the other components of the respiratory chain are restricted in the *cristae* compartment. Thanks to the development of 3D electron microscopy techniques, it has been demonstrated that *cristae* are not just invagination of the inner mitochondrial membrane as previously depicted by Palade in 1952, but distinct compartments of it, separated from the intermembrane space by narrow tubular *cristae* junctions (Mannella et al., 1994). During apoptosis, to reach a complete release of cytochrome *c* mitochondria undergo ultrastructural changes collectively called *cristae* remodelling characterized by the fusion of individual *cristae* and the widening of tubular narrow *cristae* junctions (Scorrano et al., 2002). The ultrastructural remodeling is accompanied by changes in the shape of mitochondrial network that undergoes massive fragmentation.

The shape of mitochondria is determined by the equilibrium between fusion and fission processes, controlled by a family of "mitochondria-shaping proteins", many of which are dynamin-related GTPases. In mammalian cells, mitochondrial fusion depends on mitofusin 1 and 2 in the outer mitochondrial membrane and on the inner membrane protein optic atrophy 1 (OPA1) (Olichon et al., 2002). In our laboratory it has been demonstrated that OPA1 requires mitofusin 1 in order to promote mitochondrial fusion (Cipolat et al., 2004). Fission requires the additional step of translocation of dynamin-related protein 1 (Drp1) from the cytosol to mitochondria where it presumably docks on hFis1, its adaptor in the outer membrane. Oligomerization of Drp1 is believed to provide the mechanical force to constrict mitochondrial membranes and to fragment the organelle (Hinshaw et al,, 1999; Smirnova et al, 2001). Translocation of Drp1 to mitochondria depends on its dephosphorylation by calcineurin and other phosphatases (Cereghetti et al., 2008).

A growing body of evidence suggests that mitochondria shaping proteins participate in cell death. In our laboratory it has been recently demonstrated that OPA1 protects from apoptosis by preventing cytochrome *c* release independently of mitochondrial

fusion. OPA1 keeps in check the *cristae* remodelling pathway and tightness of *cristae* junctions correlates with oligomerization of two forms of OPA1, a soluble intermembrane space and an integral inner membrane one (Frezza et al., 2006; Cipolat et al., 2006). Moreover, other works showed that the expression of a dominant negative mutant of Drp1, with disrupted enzymatic activity, prevents mitochondrial fragmentation, cytochrome *c* release and cell death induced by intrinsic apoptotic stimuli (Frank et al., 2001). Interestingly, Drp1 seems to specifically regulate the release of cytochrome *c*, suggesting that the mitochondrial subcompartmentalization of the pro-apoptotic factor can be altered by Drp1 (Parone et al., 2006).

The aim of my thesis has been to study the role of calcineurin-dependent mitochondrial fission in cell death and in the pathogenesis of Huntington's Disease.

Recently our laboratory demonstrated that mitochondrial depolarization triggers activation of the cytosolic phosphatase calcineurin. Calcineurin dephosphorylates Drp1 on Ser637, stimulating its translocation to mitochondria, and ultimately leading to the fragmentation of the organelle (Cereghetti et al., 2008). To further characterize the importance of this pathway in apoptosis we focused on a new specific inhibitor of calcineurin, a peptide, PPD1, corresponding to the peptidylproplyl isomerase domain of immunophilin FK506 binding protein 52. Besides being genetically encoded and therefore more stable than small pharmacological inhibitors, PPD1 doesn't affect the interaction of Drp1 with the dynein motor complex, also implied in the translocation of Drp1 to mitochondria, as we showed by co-immunoprecipitation experiments. Time course confocal microscopy analysis of HeLa cells transfected with a red fluorescent protein targeted to mitochondrial matrix (mtRFP) showed that expression of PPD1 strongly delays mitochondrial fragmentation induced by mitochondrial depolarization. This effect can be ascribed to the observed reduction of Drp1 translocation to mitochondria. We then wanted to check whether PPD1-dependent inhibition of fragmentation could interfere with apoptosis. To this aim, we analyzed the kinetics of cell death induced by a panoply of intrinsic apoptotic stimuli. The overexpression of PPD1 could protect from apoptosis induced by staurosporine, hydrogen peroxide, etoposide and thapsigargin. Moreover, the cytoprotective effect of PPD1 was strongly confirmed by a clonogenic assay in which cells expressing PPD1 showed higher ability in surviving and generating new colonies despite the treatment with staurosporine. To understand whether PPD1 could affect the release of cytochrome *c* we overexpressed mtRFP as marker of the mitochondrial network and then we

immunodecorated cytochrome *c* with a FITC-conjugated secondary antibody. Overexpression of PPD1 prevented cytochrome *c* release in response to staurosporine. We then wished to ascertain whether PPD1 inhibits apoptosis by blocking the DRP1 pathway. To exclude the possibility that the observed protection exerted by PPD1 was due to the inhibition of dephosphorylation of other calciuneurin substrates involved in apoptosis, like the Bcl-family protein BAD, we turned to a genetic approach. We analyzed the effect of PPD1 on apoptosis in wt and *Bad^-/-* mouse embryonic fibroblasts (MEFs). Our expreriments demonstrated that BAD is dispensable for the antiapoptotic action of PPD1, since the overexpression of the peptide could protect *Bad^-/-* MEFs from cell death induced by staurosporine. Moreover, the co-expression of PPD1 and a dominant negative mutant of Drp1 (DRP1^{K38A}) did not protect more, suggesting that they act via the same pathway. These data further support the decisive role of mitochondrial fission and the cellular cues that regulate mitochondrial shape in apoptosis (Cereghetti, Costa et al., in revision, Cell Death and Differentiation).

Increasing evidence show that in pathological conditions characterized by increased apoptosis, as in neurodegenerative diseases, mitochondria display changes in shape and ultrastructure. In particular, our attention was caught by electron microscopy images of mitochondria from lymphoblasts from Huntington's Disease patients that showed deep ultrastructural alterations strongly resembling *cristae* remodelling. Huntington's disease is a dominant genetic neurodegenerative disorder caused by the polyglutamine expansion in the huntingtin protein. The disease is characterized by specific loss of medium spiny neurons in the striatum, resulting in motor and cognitive symptoms. We therefore decided to characterize the mitochondrial morphological alterations and their effect on cell death in two different models of Huntington's Disease: immortalized lymphoblasts from patients and striatal neuronal precursors from knock-in mouse models bearing a polyglutamine expansion in the endogenous huntingtin gene. Confocal imaging analysis of mitochondrially targeted yellow fluorescent protein (mtYFP) revealed that HD cells display fragmented mitochondrial network if compared with the relative controls. Notably, the fragmentation was more severe in homozygous than in heterozygous patients, supporting a phenotype-genotype correlation. We then checked whether the morphological phenotype could be ascribed to alterations in levels of mitochondria-shaping proteins. Despite no differences in their total levels, more Drp1 was retrieved on mitochondria isolated from

HD cells than in wt. Moreover, crosslinking experiments revealed that Drp1 was more oligomerized in HD cells. These data suggested that an over-activation of the fission machinery could account for the observed fragmentation. Interestingly, OPA1 isoforms pattern at steady state and their processing upon staurosporine treatment was altered in HD cells, indicating a possible molecular mechanism through which Drp1 could regulate *cristae* morphology. We then decided to check whether we could correct the morphological phenotype either promoting mitochondrial fusion or inhibiting the mitochondrial fission machinery. To this aim, we co-transfected our cell lines with mtYFP and empty vector or OPA1, MFN1, DRP1^{K38A} and a dominant negative mutant of calcineurin. We observed that both the overexpression of pro-fusion proteins and the inhibition of the fragmentation pathway could correct the mitochondrial shape alterations. The following step was to understand which was the functional meaning of the observed morphological defects and whether they could influence apoptosis. Flow cytometric analysis of cell death of lymphoblasts revealed that cells from HD patients are more susceptible to staurosporine. The same was confirmed in neurons from HD mice, which show increased poly ADP-ribose polymerase (PARP) cleavage, an hallmark of apoptosis. Interestingly, the overexpression of OPA1 and DRP1^{K38A} or the treatment with the calcineurin inhibitor FK506 restored the apoptotic susceptibility of HD cells to wt levels. However, the overexpression of MFN1 did not confer protection towards cell death, meaning that fusion *per se* is not sufficient for counteracting apoptotic stimuli. The analysis of the kinetic of cytochrome *c* release from isolated mitochondria upon treatment with the apoptotic stimulus cBID, using a specific ELISA assay, showed that HD organelles release cytochrome *c* faster than the relative control. Moreover, crosslinking experiments demonstrated that the enhanced release of cytochrome *c* correlates with a faster disruption of OPA1 oligomers. The increased *cristae* remodelling process in HD mitochondria was further confirmed by electron microscopy imaging of mitochondrial ultrastructure upon treatment with staurosporine. HD cells showed decreased number of *cristae* per mitochondrion at steady state and the *cristae* compartment became highly deranged and reorganized following the apoptotic stimulus. Importantly, overexpression of OPA1 and DRP1^{K38A} could clearly increase the number of properly structured *cristae* and inhibit their reorganization during apoptosis.

In conclusion, the data presented in this doctoral thesis show that the calcineurin-DRP1 axis-dependent regulation of mitochondrial fragmentation plays a crucial role in

the progression of the intrinsic apoptotic cascade. Furthermore, we could demonstrate that this pathway is altered in Huntington's disease and could account for the increased cell death that characterizes this neurodegenerative disorder (Costa et al., submitted, EMBO Mol. Med.).

3 Introduction

Eukaryotic cells are surrounded by a plasma membrane (PM) and contain extensive internal membranes that enclose specific compartments, the organelles, and separate them from the rest of the cytoplasm, the region of the cell lying outside the nucleus. Most eukaryotic cells contain many mitochondria, which occupy up to 25% of the cytoplasmic volume.

Mitochondria are the main site of ATP production. In addition to supplying cellular energy, they are involved in a range of other processes, such as signalling, cellular differentiation and death, as well as the control of the cell cycle and cell growth. Finally, mitochondria have been implicated in several human diseases, including degenerative disorders and cancer and may play a role in the aging process.

Mitochondria are complex organelles and their elaborate structure is very important for their function. In certain cell types they are organized in networks of interconnected mitochondria. Ultrastructurally, the IM can be further subdivided in an inner boundary membrane and in the cristae compartment, bag-like folds of the IM connected to it via narrow tubular junctions. The ultrastructure and the reticular organization of the organelle are determined by mitochondria-shaping proteins that impinge on the equilibrium between fusion and fission processes.

We will now discuss the mechanisms involved in the regulation of mitochondrial dynamics and apoptosis. Moreover, we will focus on the role of the proteins that regulate mitochondrial morphology and ultrastructure in neurodegeneration.

3.1 Mitochondria

Mitochondria are crucial organelles in life and death of eukaryotic cells. In the last few decades their role in cellular physiology and function has been reconsidered and the number of processes in which they are involved is likely to increase in the future.

They produce most of the ATP needed for endoergonic processes and convey it to the sites of greater energy demand. They participate to and shape complex signalling processes such as cytosolic $Ca2+$ transients (Jouaville et al., 1995). During apoptosis they integrate diverse stimuli by releasing protein cofactor needed in the cytosol to grant the efficient activation of the caspases that execute cell destruction (Wang, 2001). Defects in any of these processes can be detrimental for the cell: several

diseases are consequences of, or are aggravated by, mitochondrial dysfunction (Schapira, 2006). Moreover, evidence has been accumulating in favour of a direct implication of mitochondria in oncogenesis. Cancer cells display changes in cellular metabolism (the so called Warburg effect) (WARBURG, 1956) and tend to disable the mitochondrial pathway of apoptosis (Hanahan and Weinberg, 2000). Unlike any other organelle, except for chloroplasts, mitochondria appear to originate only from other mitochondria. They contain their own circular DNA (mtDNA), along with their own transcriptional and translational machinery. Mitochondrial ribosomes and transfer RNA molecules are similar to those of bacteria, as are components of their membrane. These and related observations led L. Margulis, in the 1970s, to propose an extracellular origin for mitochondria, the endosymbiotic theory (Margulis, 1971). Mutations in mtDNA are associated with a number of genetic, multisystemic diseases that highlight the importance of this organelle in physiology of multiple organs.

3.1.1 The metabolic role of mitochondria

Pioneering biochemical studies have long forged the concept that the mitochondrion is the "energy powerhouse" of the cell. It is the centre of cellular energetic metabolism, being the principal source of ATP for eukaryotic cells: only 5% of ATP generate by glucose is provided by glycolysis. ATP is required to drive most energy-dependent intracellular processes. Due to their function, mitochondria display very well-characterized mechanisms to regulate production, consumption and conservation of cellular energy.

The three major processes leading to ATP synthesis are:

- The tricarboxylic acid cycle, located in the mitochondrial matrix, in which NADH and FADH2 are produced from organic compounds
- The mitochondrial respiratory chain, in which electrons are sequentially transferred to oxygen by electron carriers, the respiratory chain complexes. To limit free energy dissipation, electrons from NADH are transferred stepwise from the IM-associated respiratory chain complexes with higher redox potential to the ones with lower. The 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 turns shuttle them to complex IV (cytochrome c oxidase). Complex IV finally catalyzes the electron transfer from cytochrome c to O2. Electrons transfer in complexes I, III and IV is coupled to proton pumping from the matrix to the intermembrane space.

- The phosphorilating system, which uses the energy supplied by the respiratory chain to catalyze the synthesis of ATP from ADP and Pi.

In 1961 Mitchell proposed that the fundamental mechanism of energy transduction in mitochondria is chemiosmosis (Mitchell, 1979), in which the free energy of oxidation of carboxylic acids is used to pump protons from the matrix to the intermembrane space establishing an electrochemical gradient. Since the inner mitochondrial membrane displays an extremely low passive permeability to ions in general and protons in particular, the result is the buildup of a proton electrochemical gradient ($\Delta\mu H+$) across the membrane. The electrochemical gradient is the sum of two components: the proton concentration difference across the membrane (ΔpH) and the electrical potential difference across the membrane ($\Delta\psi_m$). The estimated magnitude of the proton electrochemical gradient is about -220 mV (negative inside) and under physiological conditions most of the gradient is in the form of $\Delta\psi_m$. The proton gradient is utilized to synthesize ATP: the F_1F_0-ATP synthase couples the transport of these protons back across the inner membrane into the matrix with the phosphorylation of ADP to produce ATP.

Mitochondria are the site of many other metabolic processes, such as biosynthesis and degradation of fatty acids and amino acids, and synthesis of ketone-bodies and heme. They also play a major role in generation and detoxification of reactive oxygen species and in H^+ and Ca^{2+} homeostasis

3.1.2 Mitochondrial biogenesis

Mitochondria perform essential cellular functions, yet can not be synthesized de novo (Attardi and Schatz, 1988). Instead, these organelles are derived from pre-existing mitochondria and specific cellular mechanisms act to ensure their faithful transmission to the progeny.

A growing list of key protein components of the inheritance process emerged from analysis of conditional mutants of the budding yeast Saccharomyces cerevisiae that

were defective for mitochondrial distribution and morphology (the mdm mutants) (Berger and Yaffe, 1996; Koehler et al., 1999; Hermann and Shaw, 1998).

Mitochondria consist of four distinct compartments. The mitochondrial outer and inner membranes serve as barriers for the maintenance and integrity of two soluble compartments, the mitochondrial intermembrane space (IMS) and mitochondrial matrix. The outer membrane is composed of about half lipid and half protein, contains specific transports and pores that render the membrane permeable to molecules having molecular weights as high as 10 KDa. The OMM is similar to the outer membrane of gram-negative bacteria. The inner membrane is less permeable, constituted for approx. 80% of proteins, a higher proportion than that occurring in other cellular membranes.

Mitochondria contain their DNA: in vertebrates, mtDNA consists of a double stranded covalently closed circular DNA molecule of about 16.5 kb. Many mtDNA molecules are packaged within mitochondria into small clusters called nucleoids (Jacobs et al., 2000), that vary in size and number in response to physiological conditions (Nosek and Tomaska, 2003; Legros et al., 2004; Malka et al., 2006). Nucleoid structure is stabilized by TFAM, or mtTFA, which binds to mtDNA and regulates its abundance (Kanki et al., 2004). The maintenance of the mtDNA integrity is important for keeping proper cellular functions both under physiological and pathological conditions (Kang et al., 2007).

Mitochondria contain about 1500 different proteins, only half of which have been identified (Calvo et al., 2006). mtDNA encodes 13 mRNAs for subunits of the oxidative phosphorylation complexes (OXPHOS) (Anderson, 1981; Fernandez-Silva et al., 2003). Proteins of mitochondrial origin are translated on mitochondrial ribosomes bound to the matrix side of the inner membrane, and cotranslationally inserted into the proper compartment (Allen et al., 2005; Poyton and McEwen, 1996). Ninetynine percent of the 1,000 different mitochondrial proteins are produced on cytosolic ribosomes and are imported into the organelle (Sickmann et al., 2003; Prokisch et al., 2004). The correct delivery and sorting of nuclear encoded mitochondrial precursors to each of these four compartments is pivotal in the maintenance of normal organelle function and structure. However, in addition to be delivered to the correct submitochondrial location, many proteins have to be further assembled into homo- or hetero-oligomeric structures in order to fulfill their functions. Translocation machineries

within the outer and inner membranes in addition to translocation mediators within the organelle's soluble regions exist and execute these delicate tasks (Koehler, 2004).

Nuclear-encoded mitochondrial proteins are imported by the TOM complex. Subsequently, they follow different pathways. Presequence-carrying proteins are transported by the TIM23 complex and the motor PAM into the matrix, where mitochondrial processing peptidase (MPP) cleaves off the presequences. Small proteins of the intermembrane space (IMS) are imported via the mitochondrial intermembrane space assembly machinery (MIA). β-barrel precursors of the outer membrane (OM) are transferred by the Tim9–Tim10 chaperone complex from TOM to SAM. Precursors of inner membrane (IM) carriers use Tim9–Tim10 for transfer to the TIM22 complex that drives insertion into the inner membrane.

The protein translocases in the four mitochondrial compartments do not function as independent complexes but cooperate in a dynamic manner. This includes transient contacts between translocases located in different compartments and the involvement of protein complexes that have previously been thought not to be related to protein biogenesis, such as the respiratory chain and mitochondrial morphology components.

3.1.3 Mitochondrial ultrastructure

Since the 1950s, electron microscopy (EM) has detailed the substructure of the cell. Sjöstrand and Palade led pioneering work on electron microscopy of mitochondria. They both recognized that mitochondria have two very different membranes, an outer (OMM) and a highly convoluted inner (IMM) membrane. It was proposed that mitochondria posses two soluble compartments: the intermembrane space (IMS), between the OM and the IM, and the matrix, the central electrondense space. Palade's model evolved into that currently depicted in textbooks in which the inner mitochondrial membrane is one continuous closed surface with a complex morphology, folded in ridges called cristae (Figure 1A). This model, sometimes called the baffle model, depicts the cristae with broad openings to the intermembrane space on one side of the mitochondrion and protruding across the matrix nearly to the other side (Palade, 1952).

Some years later Hackenbrock demonstrated that the structure of mitochondrial membrane is linked to the metabolic state of mitochondria. For example, the matrix "contracts" during changes in osmotic or metabolic state, producing a *condensed*

conformation in which the inner membrane is pulled away from the outer membrane except at loci he called 'contact sites' (Hackenbrock, 1966). By contrast, mitochondria observed in situ are usually found in the *orthodox* conformation, characterized by a relatively large matrix volume and the non-cristal component of the inner membrane closely apposed to the outer membrane with a small intermembrane space.

The need to reinvestigate the issue of mitochondrial compartmentation provided the impetus to apply a new 3D imaging technology, EM tomography, to this problem. In EM tomography, multiple projection images representing many different views are collected from a specimen whose thickness can range from 0.25 to 1.5 µm or more. Imaging of these thick specimens is obtained using electron microscopes operating at high voltages (400–1200 kV) equipped with precision tilting stages. The first applications of EM tomography to mitochondria were performed by Mannella and co-workers on rat-liver mitochondria (Mannella et al., 1994). Sections used were 0.5 µm thick, representing a large segment of the intact, isolated organelles, typically ellipsoids, 0.5–2 µm in size. The resulting tomograms provided striking evidence that the standard baffle model was incorrect, at least for isolated rat-liver mitochondria, which are among the most commonly used for bioenergetic investigations.

When Frey, Perkins and co-workers applied high-voltage electron tomography coupled with three-dimensional image reconstruction *in situ* in several different tissues, they identified the cristae as a novel compartment. Cristae are separated from the inner boundary membrane, shaped like bags and connected by narrow tubular junctions (with a diameter of approximately 28 nm) to the thin intermembrane space (Perkins et al., 2001) (Figure 1 B).
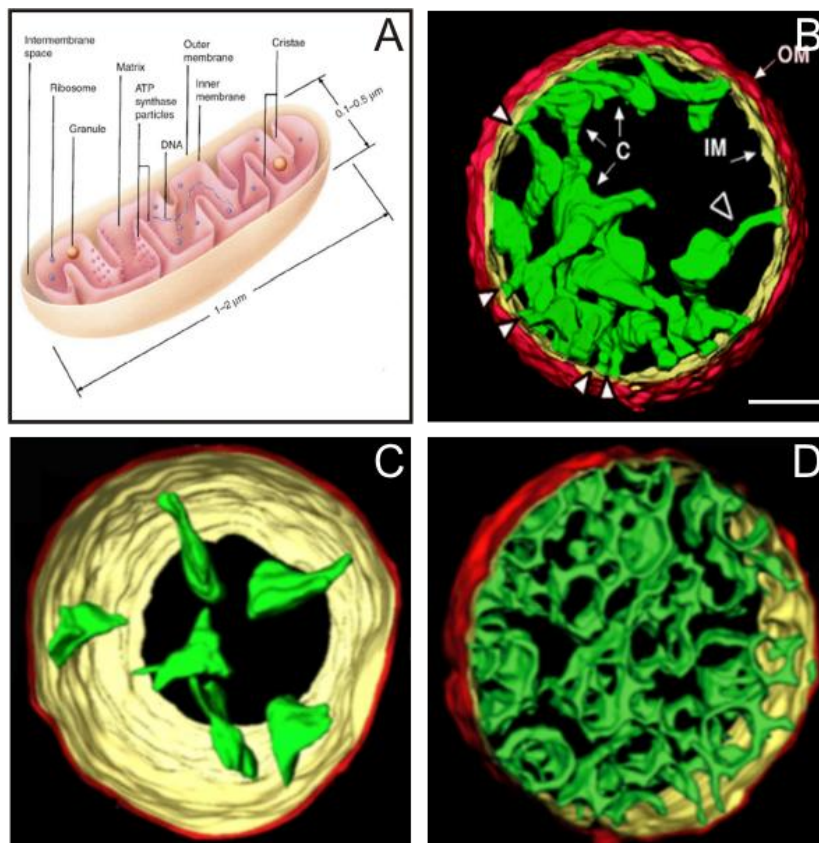
Figure 1: Mitochondrial ultrastructure. (A) A text book like representation of the buffle model adapted from (Frey and Mannella, 2000) (**B**) Three-dimensional reconstructions of isolated rat liver mitochondria obtained by high-voltage electron microscopic tomography. OM: outer membrane, IM: inner membrane, C: selected cristae; arrowheads point to narrow tubular regions that connect cristae to periphery and to each other. Bar, 0.4 μm. Adapted from (Frey and Mannella, 2000). (**C-D**) Representative surface-rendered views of electron microscopy tomography reconstructions of class I (**C**) and II mitochondria (**D**). The OM is depicted in red, the inner boundary membrane in yellow, and the cristae in green.

In contrast to the standard baffle model for mitochondrial structure, this new structural organization strongly suggest that diffusion between internal compartments is restricted, which has profound functional implications. Because oxidative phosphorylation relies on rapid diffusion of ions and substrates to sites of transport or reaction on the mitochondrial inner membrane, the number and shape (diameter and length) of cristae junctions could regulate rates of ATP phosphorylation under certain conditions (Perotti et al., 1983; D'Herde et al., 2001). Likewise, the shape and volume of cristae can be expected to affect the diffusion of cytochrome c between intracristal and intermembrane compartments (Bernardi and Azzone, 1981; Scorrano et al., 2002).

3.1.4 Mitochondrial movement and transport

Mitochondria distribution and transport inside a cell is likely to be a very well-regulated process. These organelles are often enriched at cellular sites where energy demand is greater, or where their metabolic function is required, like at the level of the synaptic button. This implies that mitochondria are (i) mobile; (ii) use cytoskeletal proteins as

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tracks for their directional movement; (iii) are transiently or permanently stopped where their presence is required via interactions with specialized cellular structures.

Mitochondrial transport depends on the actin cytoskeleton in budding yeast (Fehrenbacher et al., 2004) and on both actin and microtubules in mammalian cells (Morris and Hollenbeck, 1995; Hollenbeck and Saxton, 2005). Depending on the cellular context, these transport processes can ensure proper inheritance of mitochondria or can recruit mitochondria to active regions of the cell. For example, in budding yeast, mitochondria are transported into and retained in the developing bud to ensure mitochondrial inheritance to the daughter cell (Fehrenbacher et al., 2004).

Energy-dependent molecular motors transport mitochondria along cytoskeletal filaments. Along microtubules, multiple kinesin family members and cytoplasmic dynein have been implicated in anterograde and retrograde mitochondrial transport, respectively (Hollenbeck and Saxton, 2005).

Recent work has clarified the linkage between mitochondria and kinesin-1. Genetic screens in *D. melanogaster* identified milton and miro, both of which are required for anterograde mitochondrial transport in neurons (Guo et al., 2005; Stowers et al., 2002). Milton interacts directly with kinesin and miro, which is a mitochondrial outer membrane protein that has GTPase and Ca2+ binding EF-hand domains (Glater et al., 2006). It is very likely that miro is not only an adaptor for milton, but is also a critical regulator of kinesin-dependent mitochondrial transport: either the GTPase activity or calcium binding can regulate miro's conformation and, therefore, its ability to recruit milton or arrange the milton–kinesin complex at the surface of mitochondria. In yeast, disruption of the Miro orthologue Gem1p results in abnormalities in mitochondrial morphology and poor respiratory activity (Frederick et al., 2004). Both GTP-binding and Ca2+-binding motifs are essential for Gem1 function, which appears not to be involved in fusion or fission.

Depending on the cell type, mitochondria can also travel along actin filaments under the control of myosin motors (Hollenbeck and Saxton, 2005).

3.2 Mitochondria shape and dynamics

Mitochondria are dynamic organelles able to change number and shape in living cells during development, mitosis -when they co-ordinately divide into daughter cells (Catlett and Weisman, 2000) and in response to physiological or toxic conditions.

Mitochondrial structure is very heterogeneous and can range from small, individual spheres to interconnected and branched tubules. The different and dynamic changes in mitochondrial shapes were already noticed in early times by cytologists who observed living cells under light microscopy: due to the high heterogeneity of mitochondrial shape, they christened this organelle "mitochondrion", a combination of the Greek word for μίτος or mitos, "thread" and χονδρίον or khondrion, "grain".

In the cytosol of certain cell types, mitochondria can be organized in a net, where individual organelles dynamically fuse and divide (Legros et al., 2002) to generate functional units of fused mitochondria, where a stimulus or a signal hitting one end of the mitochondrial wire can be readily transmitted to other distal components of the net (Amchenkova et al., 1988). This property is useful to rapidly convey signals across the cytoplasm, especially in large cells such as cardiomyocytes (Pacher and Hajnoczky, 2001). On the other hand, in other cell types both shape and function of individual mitochondria are heterogeneous, with limited interconnectivity of the organelles (Collins et al., 2002).

The morphological plasticity of mitochondria results from the equilibrium between fusion and fission events. Even more remarkably, imaging studies in live cells indicate that mitochondria constantly move and undergo structural transitions. Individual mitochondria move back and forth along their long axes on radial tracks. Occasionally two distinct mitochondria can encounter each other during these movements and undergo fusion, head to head or side to head. Conversely, long tubules can divide by fission events, giving rise to two or more distinct units. Mitochondria are organelles surrounded by two membranes making the fusion and fission complicated processes. Any fusion or fission event requires the coordinate fusion-division of four lipid bilayers in a coordinate and complete manner.

Dynamic control of mitochondrial structure is performed by a growing set of "mitochondria-shaping" proteins that include both pro-fusion and pro-fission members.

3.2.1 Mitochondria-shaping proteins

Several members of the "mitochondria-shaping" protein family are dynamins-related proteins. Dynamins are ubiquitous mechano-enzymes that hydrolyze GTP to regulate fusion, fission, tubulation and elongation of cellular membranes (McNiven et al., 2000). The role of dynamins in controlling mitochondrial shape was initially identified by genetic screens in budding yeast, where deletion of specific genes resulted in gross

alterations of the mitochondrial network, and ultimately in functional abnormalities including loss of mitochondrial DNA, growth defects and petite strains (Dimmer et al., 2002; Shaw and Nunnari, 2002).

3.2.1.1 Proteins involved in mitochondrial fusion

3.2.1.1.1 Fzo/ Mitofusin-1,-2

The first mediator of mitochondrial fusion identified was the *D. melanogaster* Fuzzy onion protein (Fzo1), an evolutionarily conserved, large transmembrane GTPase localized in the outer mitochondrial membrane. In Drosophila the Fzo gene is activated during spermatogenesis and mutations in the gene are responsible for sterility in the male fly (Hales and Fuller, 1997). Fzo1 mediates the formation of two giant mitochondria (the so called Nebenkern structure) in the spermatide, required to give energy to the flagellum.

The *S. cerevisiae* orthologue of Fzo1 mediates mitochondrial fusion events during mitotic growth and mating and is it required for long-term maintenance of mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998). Fzo1p has two homologues in mammals, MFN1 and MFN2 (Santel and Fuller, 2001), which both control mitochondrial fusion. MFN1 and -2 display high (81%) homology and similar topologies, both residing in the OM (Rojo et al., 2002; Chen et al., 2003; Santel et al., 2003) They possess an N-terminal GTPase domain, two transmembrane domains spanning the outer mitochondrial membrane and two coiled coil motifs crucial for protein–protein interaction (Rojo et al., 2002; Santel and Fuller, 2001).

Despite their highly similarity, the two mitofusins display some structural and functional divergences. MFN2 possesses a p21ras-binding domain at its N-terminal, which is not retrieved in MFN1 (Chen et al., 2004). Moreover, in silico analysis of MFN2 reveals that this protein also has a proline-rich-domain between aminoacids 576 and 590, which is poorly conserved in MFN1. Proline-rich domains are involved in the binding to other proteins (Kay et al., 2000). MFN1 and MFN2 are believed to dock two juxtaposed mitochondria via their coiled coil domains (Koshiba et al., 2004). However, MFN2 seems to have a different role from MFN1. First, it has been shown that MFN1 has a higher GTPase activity than MFN2, although its affinity for GTP is lower (Ishihara et al., 2004). In agreement with this, MFN1 exhibits a higher capacity to induce fusion (Ishihara et al., 2004). The differential role played by the two mitofusins during

mitochondrial fusion was first demonstrated by directly measuring mitochondrial fusion rates in *Mfn1-/-* and *Mfn2-/-* cells. These experiments substantiated that cells containing only MFN1 retain more fusion activity than those that contain only MFN2 (Chen et al., 2003). Extending these cell biological observations, genetic ablation of the two genes in the mouse does not result in the same phenotype: *Mfn1-/-* mice die in midgestation, whereas *Mfn2-/-* embryos display deficient placentation (Chen et al., 2003).

A recent work from our lab demonstrated that MFN2 is localized not only on the outer mitochondrial membrane but also on ER and it is enriched at the ER-mitochondria interface. Besides controlling ER morphology, MFN2 on ER forms homotypic and heterotypic complexes with MFN1 and MFN2 on the outer mitochondrial membrane and it controls the tethering between the two organelles. The regulation of this juxtaposition is crucial for the mitochondrial Ca^{2+} uptake upon Ca^{2+} release from ER stores (de Brito and Scorrano, 2008).

3.2.1.1.2 Mgm1p/ Msp1p/Opa1

Optic atrophy 1 (OPA1) is a dynamin-related protein located in the IMM. Mgm1p, the yeast homologue of OPA1, has been identified in a genetic screen for nuclear genes required for the maintenance of mtDNA in the budding yeast *S. cerevisae* (Jones and Fangman, 1992). Years later, Pelloquin and colleagues isolated Msp1p, the *S. pombe* orthologue (Pelloquin et al., 1999). The human gene of Opa1 was identified in 2000 by two independent groups (Delettre et al., 2000; Alexander et al., 2000). Albeit Mgm1p and Opa1 display a sequence identity of only approx. 20%, they maintain a highly conserved secondary structure, consisting of a N-terminal mitochondrial targeting sequence (MTS) composed of scattered positively charged amino acid residues, two consecutive hydrophobic segments, a GTPase domain, a middle domain, and a C-terminal coiled-coil domain that may correspond to GTPase effector domain GED (Satoh et al., 2003). The pleckstrin homology and proline-rich domains, found in classical dynamins, are missing. The functional analysis of Mgm1p and Msp1 reveals that both proteins are required for the maintenance of fusion-competent mitochondria in *S. cerevisiae* and *pombe* (Wong et al., 2003). The high degree of secondary structure conservation suggests that the function of OPA1 is conserved in mammals. On the other hand, it was less clear whether OPA1 played a role in fission, or in fusion

of mitochondria, since high levels of OPA1 can drive fragmentation of the mitochondrial reticulum (Griparic et al., 2004; Misaka et al., 2002; Olichon et al., 2003). However, in our laboratory it has been shown that a linear relationship between OPA1 levels and mitochondrial fusion exists, as overexpression of OPA1 enhances fusion, while its downregulation by siRNA represses it in mouse embryonic fibroblasts. Moreover, a genetic approach demonstrated that OPA1 requires MFN1 on the outer mitochondrial membrane to exert its fusion effect (Cipolat et al., 2004). We will discuss later the role of OPA1 in apoptosis and in disease.

3.2.1.2 Proteins involved in mitochondrial fission

3.2.1.2.1 Dnm1p/Dlp1/Drp1

The two proteins FIS (fission) 1 and DRP1 regulate mitochondrial fission in mammals. The dynamin-like-protein (Dlp) 1p in yeast, DRP-1 in C. elegans, and DLP1/DRP1 in mammals are homologues. DRP1 exists largely in a cytosolic pool, but a fraction is found in spots on mitochondria at sites of constriction (Labrousse et al., 1999; Smirnova et al., 2001). DRP1 contains a dynamin-like-central domain and a C-terminal GTPase effector domain (GED), in addition to its N-terminal GTPase. DRP1 can oligomerize, *in vitro*, into ring-like structures and intermolecular oligomerization is observed at membrane constriction sites. Given these similarities with dynamin, DRP1 has been proposed to couple GTP hydrolysis with mitochondrial membrane constriction and fission (Hinshaw, 1999; Smirnova et al., 2001).

3.2.1.2.2 Fis1p/hFis1

Fis1p is a 17-kDa integral protein of the outer mitochondrial membrane (James et al., 2003). Its N-terminal domain is exposed to the cytoplasm and forms a tetratricopeptide (TPR)-like fold (Suzuki et al., 2003). The C-terminal domain of FIS1 possesses a predicted TM domain and a short stretch of amino acids facing the IMS. FIS1 is thought to recruit DRP1 to punctuate structures on mitochondria during mitochondrial fusion. It is therefore considered the limiting factor in the fission reaction (Stojanovski et al., 2004). Moreover it has been shown that hFis1 is a bifunctional protein which independently regulates fission and apoptosis (Alirol et al., 2006).

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3.2.1.2.3 Mff

Mff is a novel component of the mammalian mitochondrial fission machinery. Mff is a tail-anchored membrane protein located in the outer mitochondrial membrane and on peroxisomes. The hydrophobic carboxy-terminal segment serves as a membrane anchor, while the amino-terminal conserved repeats and the coiled coil domain might be involved in the interaction with other proteins. The silencing of Mff by siRNA inhibits mitochondrial fragmentation and leads to the appearance of an elongated mitochondrial network, similar to that obtained by silencing of Fis1 and DRP1. The topological and functional similarities between Mff and FIS1 suggests that they act in the same pathway, but the fact that they are not found in the same complex suggests that they fulfill different functions in the process of mitochondrial and peroxisomal fragmentation (Gandre-Babbe and van der Bliek, 2008).

3.2.1.2.4 Endophilin b1

Endophilin B1, a member of the endophilin family of fatty acid acyl transferases that participate in endocytosis, has been shown to play a role in mitochondrial fission (Karbowski et al., 2004). During endocytosis, endophilin 1 builds complexes with dynamin I, the dynamin responsible for the severing of the neck of the nascent endocytic vesicle, and provides the required lipid modification (Schmidt et al., 1999).
Endophilin B1 partially colocalizes and cofractionates with mitochondria and its downregulation by siRNA leads to changes in mitochondrial shape, as well as the formation of OM-bound structures resembling those formed by vesicles in neuronal terminals after inactivation of endophilin 1.
Members of the endophilin family, which are all Bin-Amphiphysin-Rvs(BAR)-domain proteins (like for example amphiphysin and endophilin 1), are supposed to participate in the regulation of membrane curvature, a process required for membrane scission during dynamin-mediated endocytosis (Gallop et al., 2006). However, the mechanism by which BAR-domain proteins and related components regulate membrane scission has recently been questioned. First, it has been proposed that BAR-domain proteins have an acyl transferase activity that promotes membrane fission by altering membrane curvature from positive to negative (Schmidt et al., 1999). Later studies by the laboratory of H. McMahon demonstrated that BAR-domain proteins have no fatty

acyl transferase activity as previously believed (Gallop and McMahon, 2005). The amphipathic helices of BAR domains alter membrane curvature by inserting into the phospholipids bilayers and not by displaying a fatty acyl transferase activity (Gallop et al., 2006). Similarly, endophilin B1 seems to participate in the control of the morphology of OM by altering membrane curvature. Whether this is a direct effect, or it requires the recruitment of other proteins, such as phospholipase D and/or other mitochondria-shaping proteins, remains to be elucidated.

3.2.1.2.5 Mtp18

MTP18, a nuclear-encoded mitochondrial membrane protein, is suggested to be a component required for mitochondrial fission in mammalian cells(Tondera et al., 2004; Tondera et al., 2005). MTP18 is supposed to be an intramitochondrial protein exposed to the IMS, however it is still not clear whether MTP18 is an OM or IM protein. Interestingly MTP18 is a downstream effector of phosphatidyl-inositol 3-kinase (PI3-K) signalling. It has been reported that overexpression of MTP18 leads to mitochondrial fragmentation. On the other hand, after downregulation of MTP18 levels by siRNA, mitochondria appear filamentous. Thus, MTP18 could be a regulator of mitochondrial shape that responds to activation of PI3-K, coupling morphology of the reticulum to cellular cues.

3.2.2 Mechanisms of mitochondrial fusion and fission

Several important characteristics of mitochondria make their fusion mechanism particularly intriguing. First, unlike almost all other intracellular fusion events, neither SNAREs nor the AAA-ATPase NSF have been implicated in the fusion reaction. Indeed, there are specific, dedicated mitochondrial fusion molecules, suggesting that the machinery evolved independently and is uniquely tailored for this organelle. Second, mitochondria have two membranes: therefore, the fusion of four sets of lipid bilayers must be coordinated. Third, unlike viral fusion and most SNARE-mediated fusion, mitochondrial fusion is homotypic. Finally, mitochondrial fusion it is likely to be influenced by cellular energetic demands, apoptotic stimuli and developmental cues. Taken together, these characteristics suggest that mitochondria fuse through a novel

mechanism that reflects their unique endosymbiontic origin and double membrane architecture.

Why do mitochondria continually fuse and divide? Recent studies show that these processes have important consequences for the morphology, function and distribution of mitochondria. First, fusion and fission control the shape, length and number of mitochondria. The balance between these opposing processes regulates mitochondrial morphology. Second, fusion and fission allow mitochondria to exchange lipid membranes and intramitochondrial content. Such exchange is probably crucial for maintaining the health of a mitochondrial population. When mitochondrial fusion is abolished, a large fraction of the mitochondrial population loses nucleoids (Chen et al., 2007). In addition to mtDNA, other components, such as substrates, metabolites or specific lipids, can be restored in defective mitochondria by fusion. Third, the shape of mitochondria affects the ability of cells to distribute their mitochondria to specific subcellular locations. This function is especially important in highly polarized cells, such as neurons. Mitochondrial fusion and fission affect the mitochondrial distribution in dendrites. In hippocampal neurons, mitochondria accumulate at dendritic spines following neuronal stimulation. Inhibition of mitochondrial fission causes elongation of the mitochondria and decreases the abundance of dendritic mitochondria and the density of dendritic spines. Conversely, increased fission facilitates the mobilization of dendritic mitochondria and leads to an increased spine number (Li et al., 2004). Mitochondrial dynamics appears to be important for proper mitochondrial redistribution in lymphocytes during chemotaxis (Campello et al., 2006). Finally, mitochondrial fission facilitates apoptosis by regulating the release of intermembrane-space proteins into the cytosol. Moreover mitochondria actively participate in the regulation of Ca^{2+} signalling by taking up and releasing Ca^{2+} in response to physiological, inositol triphosphate coupled agonists. This process relies on the relative position of mitochondria in the cytosol, as well as on their juxtaposition to the ER, required for the production of microdomains of high $[Ca^{2+}]$, essential for the activation of the low affinity mitochondrial Ca^{2+} uniporter (Rizzuto et al., 2000). It is therefore conceivable that changes in mitochondrial shape influence mitochondrial participation in the Ca^{2+} game. This hypothesis is substantiated by the finding that excessive fission by Drp1 blocks propagation of Ca^{2+} waves (Szabadkai et al., 2004), while Fis1 reduces refilling of ER Ca^{2+} stores, probably by impairing capacitative Ca^{2+} entry from the plasma membrane (Frieden et al., 2004). Surprisingly, Fis1 promotes higher degrees of fragmentation

than Drp1, yet its effect on the propagation of mitochondrial Ca^{2+} waves are apparently much lower. A possibility is that Drp1 has a specific but yet not characterized function on mitochondrial Ca^{2+} propagation, in addition to its effect on mitochondrial morphology.

3.2.2.1 Regulation of fission

Mitochondrial fission in mammalian cells seems to follow the same mechanism described in yeast. Like in yeast, it has been shown that DRP1 is recruited to spots on mitochondria and it seems that constriction of the membranes takes place via interaction with FIS1, since it has been shown that recombinant DRP1 and recombinant FIS1 can interact in vitro (Yoon et al., 2003). However, this association has never been shown in vivo and reduction of FIS1 levels by siRNA does not disrupt DRP1 localization to mitochondria (Lee et al., 2004). While this could argue against the possibility that Fis1 acts as the mitochondrial receptor for DRP1, it should be considered that the residual level of FIS1 could till be sufficient to recruit DRP1 to mitochondria. A growing number of studies is contributing to the characterization of the signals that induce the recruitment of DRP1 to mitochondria and the turnover of the protein.

3.2.2.1.1 Phosphorylation of DRP1

Mitochondrial division is coordinated with the cell cycle in higher eukaryotes. Recent experiments using cultured human cells showed that mitochondrial scission is induced at the onset of mitosis, leading to partial fragmentation of mitochondria (Taguchi et al., 2007). Revealing a direct link between the cell-cycle and the mitochondrial-division machinery, this burst of mitochondrial division is correlated with the cyclinB-cyclin-dependent kinase (CDK1-dependent) phosphorylation of DRP1. This is the first demonstration that the addition of a phosphate moiety to DRP1 regulates its mitochondrial fission activity and in vitro assays using purified proteins coupled with cell-culture experiments indicate that the most potent mitotic phosphorylation event occurs on a serine residue (S616) in the carboxyl-terminal GTPase-effector domain (GED) of DRP1.

Recently Cribbs and Strack identified a crucial phosphorylation site that is conserved in all metazoans DRP1 orthologues (Cribbs and Strack, 2007). Ser637 is phosphorylated by cyclic AMP-dependent protein kinase and dephosphorylated by calcineurin, and its phosphorylation state is controlled by sympathetic tone, calcium levels and cell viability. Thus, DRP1 phosphorylation at Ser 637 provides a mechanism for the integration of cAMP and calcium signals in the control of mitochondrial shape. However, the link between phosphorylation and DRP1-dependent mitochondrial shape changes is still unclear. A mechanistic explanation has been provided by a study that showed that cyclic-AMP-kinase-dependent phosphorylation of serine 637 in the GED can decrease the GTPase activity of DRP1 by inhibiting the intramolecular interactions known to increase the GTPase activity of DRP1 (Chang and Blackstone, 2007). However, in our laboratory it has been demonstrated that calcineurin-dependent DRP1 dephosphorylation at serine 637 promotes the translocation of the protein from the cytosol to mitochondria in response to rise in cytoplasmic Ca^{2+} (Cereghetti et al., 2008). During this thesis we further investigated the role of calcineurin-mediated mitochondrial fragmentation in apoptosis.

3.2.2.1.2 Sumoylation of DRP1

DRP1 has been shown to be sumoylated and to interact with SUMO1 by yeast two-hybrid(Harder et al., 2004). Sumoylation is a process that involves the covalent binding of the small protein SUMO to the substrate, protecting it from binding to ubiquitin and therefore from degradation by the proteasome (Johnson, 2004). In a recent work, DRP1 has been demonstrated to be the substrate of the mitochondrial-anchored SUMO E3 ligase MAPL (Braschi et al., 2009). SENP5, a SUMO protease, can desumoylated Drp1, and overexpression of cytosolic SENP5 can reverse SUMO-1-induced mitochondrial fragmentation (Zunino et al., 2007). Moreover, SENP5 is also a key player in cell-cycle progression (Di Bacco and Gill, 2006) and it regulates DRP1-dependent mitochondrial fragmentation in mitosis (Zunino et al., 2009). Interestingly, the conjugation of SUMO1 to DRP1 is increased during cell death and this stimulation appears to be Bax/Bak dependent, suggesting a link between the apoptotic machinery and the regulation of the biochemical properties of DRP1 (Wasiak et al., 2007).

3.2.2.1.3 Ubiquitinylation of DRP1 and MFNs: MARCH V

The ubiquitin ligase of the OM MARCH-V regulates targeting of DRP1 for degradation (Nakamura et al., 2006). The conjugation of the 76-amino acid protein ubiquitin to substrate proteins is involved in the regulation of a variety of cellular processes (Welchman et al., 2005) ranging from selective protein degradation to DNA repair (Huang and D'Andrea, 2006) and membrane protein trafficking (Staub and Rotin, 2006). Recent findings indicate that ubiquitylation plays a direct role in mitochondrial membrane remodeling (Escobar-Henriques et al., 2006; Nakamura et al., 2006; Yonashiro et al., 2006; Neutzner and Youle, 2005). The work from the Yonashiro and Nakamura groups reported that inhibition of MARCH-V activity by expression of a dominant negative mutant causes mitochondrial fragmentation. The promotion of fusion by MARCH-V is due both to regulation of MFN2 and ubiquitinylation of DRP1. However, Karbowski and colleagues subsequently reported that inhibition of MARCH-V increases elongation and interconnectivity of mitochondria and causes excessive recruitment of DRP1 to mitochondria (Karbowski et al., 2007). Thus, MARCH-V seems to be involved in the regulation of DRP1 subcellular trafficking and correct assembly at the scission sites on mitochondria.

3.2.2.2 Regulation of fusion

Fusion of mammalian mitochondria is thought to occur in a similar way as in yeast. The mammalian orthologues of Fzo1p, MFN1 and MFN2, are believed to dock two juxtaposed mitochondria via their coiled coil domains (Koshiba et al., 2004). In the case of MFNs, two molecules on opposing membranes can bind in trans to bridge mitochondria, maintaining a distance of 95 Å between the two membranes (Koshiba et al., 2004). But how is OMM fusion coordinated with IMM fusion? In yeast a multimolecular complex of Mgm1p, Ugo1p and Fzo1p apparently coordinates fusion of the two membranes. On the other hand, a mammalian orthologue of Ugo1p has not yet been identified and it is unclear whether OPA1 and MFN1 direclty interact to promote mitochondrial fusion.

Studies in intact cells showed that in higher eukaryotes an intact IMM potential is required for mitochondrial fusion, which conversely appears to be independent of a functional cytoskeleton (Legros et al., 2002; Mattenberger et al., 2003). Taking advantage of the yeast model, mitochondrial fusion has been recently recapitulated *in*

vitro. This approach dissected the fusion process into two mechanistically distinct, resolvable steps: OMM fusion and IMM fusion. OMM fusion requires homotypic trans-interactions of the Fzo1p, the proton gradient component of the inner membrane electrical potential, and low levels of GTP hydrolysis. Fusion of the IMM requires the electrical component of the inner membrane potential and high levels of GTP hydrolysis. However, time-lapse analysis of mitochondrial fusion in yeast and mammalian cells, in vivo, clearly shows that fusion of the OMM and IMM is temporally linked. These observations indicate that individual fusion machineries exist in each membrane and that they can communicate in vivo, resulting in coupled outer and inner membrane fusion (Meeusen et al., 2004).

3.2.2.2.1 Phospholipase D

Recent work reported the involvement of a novel phospholipase D isoform (mitoPLD), possessing a MTS that directs it to the external face of mitochondria, in fusion of the organelle. This lipid modifying enzyme participates in mitochondrial fusion by hydrolyzing cardiolipin to generate phosphatidic acid (Choi et al., 2006). Phosphatidic acid facilitates vescicular fusion driven by specialized SNARE-complexes. This indicates for the first time the existence of a common mechanism between SNARE-mediate vesicle fusion and MFN-mediated mitochondrial fusion.

3.2.2.2.2 Mitofusin Binding Protein

Mitofusin-binding protein (MIB) regulates mitochondrial morphology via its interaction with MFN1 (Eura et al., 2006). MIB is a member of the medium.chain dehydrogenase/reductase protein superfamily and has a conserved coenzyme binding domain (CBD). MIB needs an intact CBD domain to interact with MFN1, this interaction results in inhibition of MFN1 function and mitochondrial fragmentation. Only 50% of MIB is associated with mitochondria, the rest is in the cytosol or seems to be on microsomes. However, the association of MIB with other membranes does not seem to affect their morphology.

3.2.2.2.3 Degradation of mitofusins

The yeast mitofusin, Fzo1, and likely Mfn1 and 2, is regulated by ubiquitination. During mating, yeast mitochondria fragment and Fzo1 levels decrease. Interestingly, this loss

of Fzo1 is attenuated by chemical inhibition (Neutzner and Youle, 2005) or genetic inactivation (Escobar-Henriques et al., 2006) of the proteasome. During vegetative growth, Fzo1 has been shown to be ubiquitinated on Lys 48 (Neutzner et al., 2007), suggesting a proteasome-mediated degradative process on the outer mitochondrial membrane. This resembles the ER-associated degradation (ERAD) pathway of membrane-spanning protein removal and degradation. A recent work from Cohen and colleagues confirmed that the degradation of Fzo1 by the 26S proteasome is regulated by the complex composed by the F-box Mdm30 protein and Sk1-Cullin-F-box ubiquitin ligases (Cohen et al., 2008). Expression levels of mammalian MFN1 and MFN2 can be increased by treating cells with proteasome inhibitors (Karbowski et al., 2007), suggesting that the ubiquitin proteasome pathway also regulates mitofusins in mammals.

3.2.2.2.4 BAX and BAK and mitochondrial morphogenesis

The proapoptotic BCL-2 family members BAX and BAK seem to play an additional role during life of the cell in controlling mitochondrial fusion. They are retrieved in a high-molecular weight complex with MFN2 and their ablation reduces the rate of mitochondrial fusion. Moreover, in Bax/Bak knock out cells the inhibition of DRP1 activity does not promote elongation of mitochondria, indicating that BAX and BAK activate mitochondrial fusion without interfering with the normal fission process. Consistent with a role of Bax and Bak in the regulation of Mitofusins activity, the presence of Bax and Bak has been shown to alter the assembly, mobility and distribution of Mfn2 complexes in healthy cells (Karbowski et al., 2006) and BAK affinity for MFN1 and MFN2 changes during apoptosis, likely contributing to mitochondrial fission during cell death (Brooks et al., 2007).

3.2.2.2.5 OPA1 processing

A further crucial level of control is exerted by the proteolytic cleavage of Mgm1/OPA1. In yeast, at steady state, Mgm1p exists in a long l-Mgm1p and a short s-Mgm1p form (Esser et al., 2002; Herlan et al., 2003) that results from the proteolytic cleavage of the long form by the rhomboid proteases Pcp1p (Herlan et al., 2003; McQuibban et al.,

2003; Sesaki et al., 2003a). Both l-Mgm1p and s-Mgm1p are necessary for mitochondrial fusion, and deletion of Rbd1/Pcp1 results in loss of fusion activity.

The regulation of OPA1 processing in mammalian cells is matter of intense studies: this field is complicated by the fact that in human cells OPA1 is present in 8 alternatively spliced isoforms (Delettre et al., 2001). Opa1 variants are subject to a complex post-translational cleavage, represented experimentally by five bands on a Western Blot. Two higher molecular weight bands represent proteins integrated into the IMM, whereas three lower MW bands reflect forms that can be released into the IMS (Duvezin-Caubet et al., 2007).

Several groups proposed different proteases to be involved in OPA1 processing. Mihara and colleagues demonstrated that in mammalian cells the m-AAA protease paraplegin is involved in a metal-binding site-dependent processing of OPA1 (Ishihara et al., 2006). Paraplegin is an ATP-dependent metallo-protease located in the mitochondrial inner membrane with its catalytic site exposed to the matrix (Ishihara et al., 2006). Dissipation of membrane potential, paraplegin overexpression, or induction of apoptosis, stimulated OPA1 processing along with mitochondrial fragmentation (produced by cleavage at S1, in exon 5, or S2 in exon 5b).

More recently, Duvezin-Caubet and collaborators have reconstituted Opa1 processing in yeast. They demonstrated that homo-oligomeric m-AAA protease complexes composed of murine AFG3L1, AFG3L2, or human Afg3l2 subunits cleaved Opa1 with higher efficiency than paraplegin-containing m-AAA proteases, and Opa1 processing proceeded normally in murine cell lines lacking paraplegin. Notably, certain Opa1 processing products are preferentially formed depending on the splice variant analyzed and on the subunit composition of the m-AAA protease (Langer et al., 2001). Tissue-specific differences in the subunit composition of m-AAA protease isozymes as well as in the expression of OPA1 isoforms could explain why deficiencies in paraplegin in mouse and human do result in cell type specific mitochondrial defects.

Using Opa1-null cells, the group of D. Chan showed that only Opa1 mRNA splice forms that generate a long form in addition to one or more short forms support substantial mitochondrial fusion activity. By themselves, long and short Opa1 forms have little activity, but, when coexpressed they functionally complement each other. Loss of mitochondrial membrane potential destabilizes the long isoforms and enhances the cleavage of Opa1, regulated by the i-AAA protease Yme1L (Song et al., 2007). These data were confirmed by Rojo and collaborators in a recent paper, where

they showed that metalloprotease-mediated processing of Opa1 is modulated by the inner membrane potential and is likely mediated by the YME1L protease (Guillery et al., 2008).

More recently, a role for prohibitins (Merkwirth et al., 2008) and stomatin-like protein 2 (Tondera et al., 2009) in the regulation of OPA1 cleavage has been proposed. Prohibitins are membrane proteins found also in the IMM with a scaffolding function. The ablation of prohibitins causes an excessive cleavage of OPA1 with a consequent fragmentation of the mitochondrial network. Interestingly, the work of Tondera and colleagues demonstrated that the processing of OPA1 is crucial also in stress-response conditions. Indeed, the authors observed that upon specific stresses, the mitochondrial network elongates and the hyperfusion confers on cells a resistance to stress. The long form of OPA1 is necessary and sufficient to promote the stress-induced hyperfusion and the long form maintenance during stress is ensured by SLP-2, a mitochondrial inner membrane protein, member of the SPFH-family (prohibitin/ stomatin/ flotillin/ Hflk) with putative scaffolding function (Tondera et al., 2009).

The issue of OPA1 processing is crucial if one thinks that OPA1 could be a central molecular player linking mitochondrial dysfunction with changes in mitochondrial morphology. Dissipation of the mitochondrial membrane potential leads to fast induction of proteolytic processing of Opa1 and concomitant fragmentation of mitochondria. Proteolysis of Opa1 is observed in patients and in various model systems of human disorders associated with mitochondrial dysfunction: in cybrid cells from a patient with myoclonus epilepsy and ragged-red fibers syndrome, in mouse embryonic fibroblasts harbouring an error-prone mitochondrial mtDNA polymerase γ, in heart tissue derived from heart-specific TFAM knock-out mice and in skeletal muscles from patients suffering from mitochondrial myopathies (Duvezin-Caubet et al., 2006). In principle, mitochondrial dysfunction and depletion of mitochondrial ATP levels could lead to Opa1 processing, inhibition of mitochondrial fusion, and therefore to segregation of damaged mitochondria from the network of intact mitochondria (Duvezin-Caubet et al., 2006; Baricault et al., 2007).

In our laboratory it has been demonstrated that OPA1 is a substrate of PARL, the mammalian orthologue of Pcp1p (Cipolat et al., 2006). Presenilin-associated rhomboid-like (PARL), originally identified in a yeast two-hybrid screen as an interactor of presenilin (Pellegrini et al., 2001), is an inner mitochondrial membrane protease of the rhomboid family, characterized by the ability of cleaving proteins in the

transmembrane domain. The PARL-mediated OPA1 processing results in the generation of a form of the protein soluble in the intermembrane space. The processing is not related to the regulation of the prototypical function of OPA1 in fusion, but to its role in apoptosis (Frezza et al., 2006). Indeed, Parl-/- mitochondria undergo faster apoptotic cristae remodelling and cytochrome c release. The role of OPA1 in apoptosis and mitochondrial cristae shape control will be discusse more in detail in the section 3.3.3.1.1.

3.3 Apoptosis

The term apoptosis was first coined by Currie and colleagues in 1972 to describe a common, conserved, programmed type of cell death that the authors repeatedly observed in various tissues and cell types, morphological distinct from necrotic cell death (Kerr et al., 1972).

In multicellular organisms, apoptosis ensures the precise and orderly elimination of surplus or damaged cells. Cell death during embryonic development is essential for successful organogenesis and crafting of complex multicellular tissues: elimination of the webbing between digits in humans and mice, and the deletion of mammary tissue in males are good examples of this. During adulthood, it ensures the maintenance of normal cellular homeostasis and regulation of immunity. Conditions that increase or decrease normal cell death levels in different tissues can result in disease: insufficient apoptosis manifests as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases (Meier et al., 2000; Kroemer and Zitvogel, 2007; Kerr et al., 1972).

The fact that apoptosis is characterized by a stereotyped series of morphological and biochemical changes (Krammer, 2000) suggests that a common intracellular pathway ultimately leads to cell demise in a coordinated fashion, independently of the specific activating event. Depending on the cell type and the death stimulus, two main apoptotic pathways are activated: the intrinsic pathway, which is triggered by intracellular apoptotic signals and is almost always mediated by mitochondria; and the extrinsic pathway, which is triggered by activation of specific death receptors at the cell surface [reviewed in (Hacker, 2000)], and leads to pro-caspase 8 recruitment and activation. Caspase 8 can cleave other caspases (type I cells), or generate truncated Bid (tBid) that in turn triggers the mitochondrial amplification loop (type II cells).

3.3.1 Mitochondrial involvement in apoptosis

Mitochondrial involvement in apoptosis has been well characterized in the last 10 years. Its two main features include the release of proteins from the intermembrane space (IMS) and the initiation of a programme of dysfunction that includes the loss of the proton electrochemical gradient across the inner membrane. Molecular mechanisms are still under investigation and probably crosstalk mediate these two events (Bernardi et al., 2001).

The mitochondrion is at the core of the intrinsic apoptosis pathway, and provides a reservoir for protein factors that induce caspase activation. Mitochondria release into the cytosol a plethora of pro-apoptotic factors, such as cytochrome c, Smac/DABLO and Omi/HtrA2, which inhibit the cytosolic inhibitor of apoptosis proteins (IAPs) responsible for caspase inhibition; AIF and endonuclease G, which migrate in the nucleus to induce chromatin cleavage and condensation (Kroemer and Zitvogel, 2007). The most studied protein cofactor released by mitochondria in response to an apoptotic stimulus is cytochrome c (Liu et al., 1996). Nevertheless, the precise mechanism whereby cytochrome c and other mitochondrial intermembrane space proteins are released is still under active investigation.

Several hypothesis have been proposed to explain the egress of cytochrome c from mitochondria; these mechanisms may either function on their own or in cooperation: (i) in response to an apoptotic stimulus, the "BH3-only" subset of proapoptotic BCL-2 family members senses death signals and transmits them to the "multidomain" proapoptotics like Bax and Bak. These ultimately oligomerize in the mitochondrial outer membrane and form pores permitting the physical efflux of cytochrome c and of the other mitochondrial apoptotic cofactors. (ii) proapoptotic BCL-2 family members interact with intrinsic proteins of mitochondria, triggering mitochondrial dysfunction and permeability transition. (iii) Mitochondrial membrane permeabilization may result from an alteration in membrane curvature or from the formation of lipidic pores in the OM. Anyway, permeabilization of the MOM alone results only in the partial release of cytochrome c into the cytosol: the complete release of cytochrome c occurring during apoptosis requires the structural remodelling of the mitochondrial cristae.

Independently from the precise mechanism of cytochrome c egress from mitochondria, the BCL-2 family proteins appear as critical death regulators. This family of proteins, consisting of both pro- and antiapoptotic members, possesses conserved □-helices

with sequence conservation clustered in BCL-2 homology (BH) domains. Antiapoptotic members exhibit the homology in all segments from BH1 to 4, while proapoptotic molecules lack stringent sequence conservation of the first α-helical BH4 domain. Proapoptotic molecules can be further subdivided into multidomain and BH3-only proteins. Multidomain proapoptotic members such as BAX and BAK display sequence conservation in BH1-3 domains. BH3- only members display sequence conservation solely in the amphipathic α -helical BH3 region (Scorrano and Korsmeyer, 2003). Nowadays it is clear that the decision to die or not depends on the balance resulting from the activation of proapoptotic and antiapoptotic members of the BCL-2 family (Danial and Korsmeyer, 2004).

In a widely accepted model, the BH3-only members connect upstream proapoptotic signals to the mitochondrial pathway. Once activated, BH3-only proteins function as ligands for the multidomain proapoptotics BAX and BAK, induce their homo/heterooligomerization and ultimately release cytochrome c from mitochondria (Scorrano and Korsmeyer, 2003). As mentioned above, other mechanisms (for example: permeability transition or alteration of membrane curvature) may cooperate with multidomain proapoptotics during cytochrome c release.

While such a model appears substantiated by several evidences, how antiapoptotic BCL-2 family members oppose their proapoptotic counterparts is less clear. It has been proposed that BCL-2 and BCL-XL sequester BH3-only molecules, preventing activation of the multidomain proapoptotics (Cheng et al., 2001). Alternatively, antiapoptotic proteins could keep BAX and BAK in an inactive conformation, antagonizing binding to the incoming BH3-only proteins (Willis et al., 2005).

Once released, cytosolic cytochrome c binds to APAF-1, increasing its affinity for dATP/ATP. The complex composed by APAF-1, cytochrome c, dATP and ATP forms the apoptosome. The apoptosome is able to recruit procaspase-9, facilitate its auto-activation and subsequently leads to the activation of downstream executioner caspases, cysteine proteases, that effect cell demise (Zou et al., 1999; Rodriguez and Lazebnik, 1999). Executioner caspases then cleave other intracellular substrates leading to the characteristic morphological changes in apoptosis such as chromatin condensation, nucloesomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine (PS) and formation of apoptotic bodies (Hengartner, 2000).

3.3.2 Mitochondrial network morphology and apoptosis

Early in the course of programmed cell death, mammalian mitochondria coordinately fragment and undergo a reorganization of their cristae, called "cristae remodeling", in order to release most of their cytochrome c content (Frank et al., 2001; Scorrano et al., 2002; Germain et al., 2005). Mitochondrial fission is an early trait of apoptosis, occurring simultaneously to BAX translocation from the cytosol, and before activation of caspases (Frank et al., 2001). Although mitochondrial fragmentation is a common event during cell death, it is not yet clear whether fragmentation of the mitochondrial network is necessary and sufficient for induction of cell death. The fragmented appearance of mitochondria is caused by a combination of activation of the fission machinery and inhibition of the fusion one(Lee et al., 2004). The importance of mitochondria-shaping proteins and of mitochondrial shape changes in regulating mammalian apoptosis is substantiated by genetic evidences showing that inhibition of fission, as well as promotion of fusion counteract apoptosis by intrinsic, mitochondria utilizing stimuli [reviewed in (Youle and Karbowski, 2005)]. The dynamin-related protein Drp-1 and endophilin B1, a fatty acil transferase, translocate from the cytosol to foci on mitochondria early during apoptosis and they probably mediate mitochondrial apoptotic fission (Frank et al., 2001; Germain et al., 2005; Breckenridge et al., 2003). On the other hand, apoptosis can be inhibited by antagonizing the fission process. The down regulation of Drp-1 activity by RNAi or by overexpression of a dominant negative mutant Drp-1K38A, inhibits apoptosis and mitochondrial fragmentation, although Bax did translocate to mitochondria and coalesce into foci with Bak (Karbowski and Youle, 2003; Frank et al., 2001). Inhibition of FIS1 also protects from apoptosis , while its overexpression induced apoptosis (James et al., 2003; Alirol et al., 2006).
Recent works show that early in the course of cell death, MFN1 mediated fusion is suppressed and the concomitant overexpression of both mitofusins protects from apoptosis induced by intrinsic stimuli like BID and etoposide (Sugioka et al., 2004). Down regulation of OPA1 levels induces fragmentation of mitochondrial network, release of cytochrome c and apoptosis (Olichon et al., 2003).

3.3.3 Apoptotic cristae remodelling and cytochrome c mobilization

As mentioned above, the permeabilization of the OMM by BAX and BAK activation alone results only in the partial release of cytochrome c in the cytosol. Additional steps as the fragmentation of the mitochondrial network (Frank et al., 2001) and the remodelling of the cristae characterized by fusion of individual cristae and opening of the cristae junctions are required to grant the complete release of the proapoptotic factor (Scorrano et al., 2002).

Following several death stimuli, including the BH3-only protein BID (Scorrano et al., 2002), BIK (Germain et al., 2005), BIM (Yamaguchi et al., 2008) or after Fas pathway activation (Mootha et al., 2001), mitochondria remodel their internal structure and cytochrome c is mobilized from the intra-cristae compartment toward the IMS for its subsequent release across the OMM. tBID induces transient openings of the PTP and the coordinated cristae remodelling has been proved BH3 independent, BAK independent, yet CsA inhibitable (Scorrano et al., 2002) (Figure 1 C, D).

Cytochrome c is a water-soluble basic protein that is present as loosely and tightly bound pools attached to the inner membrane by its association with the anionic phospholipid cardiolipin. This association facilitates electron transport between complexes III and IV of the respiratory chain and the binding is specific and stoichiometric (Tuominen et al., 2002). During apoptosis, the detachment of cytochrome c from its high affinity cardiolipin binding sites is crucial for a complete release of the protein (Ott et al., 2002).

3.3.3.1 Factors that affect the shape of cristae

Several proteins have been implied in the regulation of the IMM topology. In the buddying yeast, mitochondrial F1FoATP synthase, is essential for normal cristae structure (Paumard et al., 2002). This role in inner membrane structure involves a dimeric form of ATP synthase that contains the additional subunits ε and γ. As visualized by electron microscopy, the ATP synthase dimer has a dimeric interface with a sharp angle that could distort the local lipid membrane. This distortion might contribute to the high membrane curvature that characterizes cristae tubules (Dudkina et al., 2005; Minauro-Sanmiguel et al., 2005). Along this line, Mgm1p is required for the oligomerization of ATP synthase, suggesting a link between these two proteins in mitochondrial morphology (Amutha et al., 2004).

Another protein involved in cristae shape regulation is Mitofilin. Mitofilin, also known as heart muscle protein, is a 90 kDa inner-membrane protein, with predicted membrane anchor and coiled-coil domains with an amino-terminal transmembrane domain, while the majority of the protein is extruding into the intermembrane space (Gieffers et al., 1997). John et al. have reported that down-regulation of mitofilin in HeLa cells by siRNA results in the formation of concentric onion-like inner mitochondrial membranes (John et al., 2005). The larger IMM structures were found to be composed of a complex, interconnected network of membranes totally lacking tubular connection to each other or to the peripheral inner membrane. Thus, the authors have proposed that mitofilin's physiological role is to maintain normal cristae morphology, in particular, the formation or stabilization of cristae junctions. Recently, the group of Capaldi demonstrated that mitofilin resides in a mitochondrial complex spanning from the IMM to the OMM that can have an implication in regulating protein import (Xie et al., 2007).

3.3.3.1.1 The role of OPA1 in cristae remodelling

Studies from our laboratory demonstrated that OPA1 is a crucial player in the regulation of cristae shape and remodeling. OPA1 is the only member of the dynamin-related protein family known so far to be resident in the IMM and this suggested OPA1 as the natural candidate to control IMM shape.

Early studies showed that the mitochondrial fragmentation caused by down regulation of Opa1 was accompanied by organelle dysfunction and cytochrome c release. Interestingly, EM analysis of OPA1-depleted cells suggested that this protein may have a role in cristae maintenance (Griparic et al., 2004; Olichon et al., 2003; Sesaki et al., 2003b; Sesaki et al., 2003a) since disorganized cristae with irregular shape, some of which showed large cristae junctions were observed. A possible role of OPA1/Mgm1 in structuring cristae is consistent with its mitochondrial localization which is basically in the cristae, as confirmed by biochemical (Griparic et al., 2004; Olichon et al., 2003) (Pelloquin et al., 1999; Wong et al., 2000) and immunogold staining (Vogel et al., 2006), even if it is not clear whether it concentrates at the cristae junction.

The evidence of a role of OPA1 in apoptotic cristae remodelling has been provided by a recent work from our laboratory. Cristae junctions are held together by OPA1 oligomers formed by integral inner membrane long forms and more soluble PARL-processed short forms. Incubating isolated mitochondria with BID induces a disruption of OPA1 oligomers and alters cristae morphology (Frezza et al., 2006). Furthermore,

the Bid-induced increase of cytochrome c release into the intermembrane space from within the cristae is blocked by overexpression of OPA1, whereas expression of Opa1 mutated in the GTPase domain increases the width of cristae junctions. Interestingly, OPA1 efficiently protects cells lacking Mfn1, essential for OPA1-mediated mitochondrial fusion (Cipolat et al., 2004) and doubly Mfn null MEFs where fusion is completely abolished (Chen et al., 2003). Thus, OPA1 has genetically and molecularly distinct functions in mitochondrial fusion and cristae remodelling during apoptosis.

The importance of a correct proteolytic processing of OPA1 in its role in cristae shape maintenance has been confirmed by a study from Merkwirth and colleagues that identified Prohibitin (Phb) 1 and 2 as regulators of OPA1 cleavage. Phb1 and 2 are membrane proteins located in the nucleus, plasma membrane and IMM with a potential scaffolding function. The ablation of prohibitins causes an impaired processing of OPA1 with the accumulation of short forms and disappearance of the long forms that leads to fragmented mitochondrial network and aberrant cristae morphogenesis. Moreover, the presence of altered cristae correlates with enhanced sensitivity to apoptotic stimuli and increased cytochrome c release (Merkwirth et al., 2008).

Interestingly, a study by the group of Shore showed DRP1-dependent remodeling of the IM during apoptosis, demonstrating for the first time a connection between mitochondrial fragmentation and cristae remodeling (Germain et al., 2005). More recently, Mopert and colleagues demonstrated that the inhibition of DRP1 expression by siRNA, besides inducing the formation of a highly branched mitochondrial network along with "bulge"-like structures, causes changes in the levels of Mitofusins and more importantly modifies the proteolytic processing of OPA1. The increase of short forms of OPA1 could correlate with the formation of bulges characterized by accumulated intramitochondrial cytochrome c deposits (Mopert et al., 2009). Whether OPA1 processing alterations are a direct cause of the imbalance of the fission-fusion turnover or result from the mitochondrial dysfunctions induced by silencing of DRP1 (Parone et al., 2008a; Benard et al., 2007) is still unknown. However, these results suggest that OPA1 can work as a molecular link between mitochondrial network morphology, organelle dysfunction and inner mitochondrial membrane cristae structure.

3.4 Mitochondrial dynamics and neurodegeneration

The nervous system is characterized by high energy demands to maintain proper neuronal functions such as axonal and dendritic transport, plasma membrane potential maintenance and release and reuptake of neurotransmitters at the synapses (Chan, 2006). Mitochondrial function is essential to neuronal processes since, unlike other cell types, neurons cannot switch to glycolysis when oxidative phosphorylation is impaired. Therefore, it is not so surprising that mutations in proteins regulating mitochondrial shape and transport contribute to neurodegenerative diseases (Glater et al., 2006). Moreover, recent findings suggest that in other common hereditary and sporadic neuropathies, such as Parkinson's (Mandemakers et al., 2007) and Alzheimer Disease (Wang et al., 2009b; Cho et al., 2009), mitochondrial functions and shape are altered.

3.4.1 Mitochondrial dynamics in neurons

Synaptic function and maintenance are crucial for neuronal survival and efficient cell-to-cell communication. In this regard, mitochondria are highly relevant since synapses have high energy and calcium buffering demands (Jonas, 2006). Several studies indicate that mitochondrial fission is necessary for synaptic maintenance and plasticity. DRP1 overexpression in hippocampal neurons from rat embryos promoted synaptogenesis, whereas overexpression of the pro-fusion OPA1 and of a dominant negative mutant of DRP1 blocked it, probably by affecting mitochondrial distribution, as indicated by the inhibition of the redistribution and the extension of mitochondria into the active dendritic protrusions induced by the stimulation of synapses (Li et al., 2004). Similarly, Bcl-X$_L$ increases synapse number and function by promoting GTPase activity of DRP1 (and thus DRP1 mediated fission) in cultured hippocampal neurons (Li et al., 2008). These results have been definitely confirmed by the observation that primary cultures of neurons from the forebrain of conditional Drp1 knock out mouse showed a decreased number of neurites and defective synapses formation, likely due to the aggregation of mitochondria in the cell body (Ishihara et al., 2009). On the functional side, expression of a dominant negative Drp1 in Drosophila prevented the mobilization of the neurotransmitter reserve pool, substantiating the role of mitochondria in the proper function of synapses (Verstreken et al., 2005; Rikhy et al., 2007).

Finally, an appropriate balance of mitochondrial fusion and fission events is also necessary to maintain and protect mtDNA (Rapaport et al., 1998; Nakada et al., 2001; Ono et al., 2001; Parone et al., 2008b). This function appears to be particularly

important for neurons, since mtDNA mutations are known to accumulate in the brain with age (Corral-Debrinski et al., 1992) and they are a common feature of neurodegenerative diseases.

3.4.2 Mutations in mitochondria-shaping proteins and neurodegeneration

3.4.2.1 Opa1 and ADOA

Mutations of OPA1 are associated with autosomal dominant optic atrophy (ADOA), also known as type I Kjer disease, the most common form of inherited optic neuropathy, with an estimated prevalence of 1:50000 (Alexander et al., 2000; Delettre et al., 2000). Linkage analysis has revealed that OPA1, mapping to 3q28-q29, is the major locus (Votruba et al., 1997) and a positional cloning approach similarly identified this gene as being responsible for OPA1-type DOA (Alexander et al., 2000).

The clinical features of ADOA are the decrease in visual acuity, tritanopia (dyschromatopsia characterized by confusion in the blue-yellow hues), sensitivity loss in the central visual fields, and pallor of the optic nerve (Votruba et al., 1998; Ferre et al., 2005a). Classic DOA usually begins before 10 years of age, with a large variability in the severity of clinical expression, which may range from non-penetrant unaffected cases up to very severe, early onset cases, even within the same family carrying the same molecular defect (Delettre et al., 2002; Carelli et al., 2004). Histopathology studies have shown diffuse atrophy of the ganglion cell layer that predominates in the central retina and loss of myelin and nerve tissue within the optic nerve (Kjer et al., 1996).

Most of the mutations associated with ADOA cluster in the GTPase and in the coiled coil domain of Opa1 (Ferre et al., 2005b). Almost 50% of mutations in the OPA1 gene described to date are predicted to lead to a truncated protein and suggest that haploinsufficiency is the cause of the disease (Marchbank et al., 2002; Pesch et al., 2001). Nearly 40% of mutations occurs in the GTPase domain and may cause a dominant negative effect, impairing the mechanoenzymatic activity of the protein complexes. This possibility is reinforced by the fact that two mouse models of Opa1 mutation (a GTPase missense and a nonsense, truncative mutation) display minimal retinal defects in the heterozygous status (Alavi et al., 2007; Davies et al., 2007).

It remains unclear why Opa1-ADOA manifests with an apparently restricted clinical ocular phenotype, comprising RGC loss. Opa1 is ubiquitously expressed throughout the body: in the heart, skeletal muscle, liver, testis, and most abundantly in the brain and retina. In the human retina, Opa1 is present in the cells of the RGC layer, nerve fibre layer, the photoreceptor layer, and the inner and outer plexiform layers (IPL & OPL). A plausible hypothesis as to why RGC neurons may be more vulnerable to OPA1 inactivation could be a particular susceptibility to mitochondrial membrane disorders inducing mitochondrial dysfunction or mislocalization. Indeed, reports describe altered mitochondrial ATP synthesis and respiration in OPA1-inactivated cells (Amati-Bonneau et al., 2005a; Chen et al., 2005). Moreover, recent studies show the effect of mitochondrial morphology regulation on mitochondrial distribution in neurons and their contribution to dendrite formation and synaptic plasticity (Li et al., 2004). This could be of particular importance in RGC neurons that display a specific distribution of mitochondria in the cell body, myelinated and unmyelinated axons (Andrews et al., 1999; Bristow et al., 2002; Wang et al., 2003). Additionally, the defects in ADOA can be ascribed to the loss of the crucial control exerted by OPA1 on the structural organization of the cristae and apoptosis (Arnoult et al., 2005; Griparic et al., 2004; Lee et al., 2004; Olichon et al., 2003; Frezza et al., 2006). It is worthy to note that miss-sense point mutations in the highly conserved GTPase domain are responsible for a syndromic form of DOA associated with sensori-neural deafness, ataxia, axonal sensory-motor polyneuropathy, chronic progressive external ophthalmoplegia and mitochondrial myopathy (Amati-Bonneau et al., 2005b). Moreover, increasing evidence show a subclinical involvement of non-neuronal tissues, characterized by defects in mitochondrial dynamics (Delettre et al., 2000; Spinazzi et al., 2008), function (Lodi et al., 2004) and mtDNA content, thus revealing an unrecognized role of the Opa1 protein in mtDNA stability.

3.4.2.2 Mfn2 and Charcot-Marie Tooth type 2A

In 2004, work by Zuchner et al. mapped the mutations responsible for Charcot-Marie-Tooth 2A (CMT2A), and identified MFN2 as being the gene responsible for the disorder. CMT is one of the most common inherited disorders in humans, with an estimated prevalence of one in 2500 individuals. CMT neuropathies can be divided into 2 main groups, type 1 and type 2. In CMT1, nerve conduction velocities are

considerably reduced. In CMT2, the nerve conduction velocities are normal but conduction amplitudes are decreased, due to the loss of nerve fibres (Zuchner et al., 2004).

CMT2A is a neurodegenerative disorder characterized by the loss of sensory and motor axons at early stages of the disease and resulting in the degeneration of the neurons themselves during a later stage of the disease. The clinical symptoms of CMT are distal weakness of the lower limbs, sensory loss, decreased reflexes and foot deformities. Other symptoms include cranial nerve involvement, scoliosis, vocal cord paresis and glaucoma (Lawson et al., 2005). Mutations in MFN2 account for around 20% of CMT2 cases, making this the most prevalent axonal form of CMT. Most MFN2 mutations in CMT2A cluster within the GTPase and the RAS-binding domains and are missense mutations (Zuchner et al., 2004; Lawson et al., 2005; Kijima et al., 2005). Recently, a de novo truncation mutation in MFN2 has been associated to CMT2 and optic atrophy (also known as hereditary motor and sensory neuropathy VI, HMSN VI) (Zuchner and Vance, 2006).

Mfn2 is up-regulated in the skeletal muscle of obese patients (Bach et al., 2003) and its over-expression suppresses vascular muscle cells proliferation in animal models of hypertension via sequestration of p21RAS, inhibition of the ERK/MAPK cascade and cell cycle arrest (Chen et al., 2004).

3.4.2.3 Drp1

To date just one clinical case involving mutations in the gene coding for DRP1 has been described (Waterham et al., 2007). The patient showed microcephaly, abnormal brain development and optic atrophy at birth and he died at 1 month of age. The elevated lactate concentrations in blood and cerebrospinal fluid suggested a compromised function of mitochondria and accumulation of cerotic acid in plasma suggested a defect in peroxisomal beta-oxidation. The disorder is caused by an heterozygous mutation (A395D) in the middle domain of DRP1, that acts in a dominant-negative manner, likely interfering with DRP1 homo-oligomerization. Consistently with the role of DRP1 in the regulation of mitochondrial and peroxisomal shape (Koch et al., 2003; Li and Gould, 2003; Koch et al., 2005), fibroblasts form the patient display abnormally elongated mitochondria and peroxisomes. Several of the observed abnormalities were similar to CMT neuropathy (e.g., truncal hypotonia and

lack of tendon reflexes) and ADOA (e.g., optic atrophy) but the clinical course was more severe, suggesting that defects in mitochondrial fission have more severe consequences that alterations in fusion and that the clinical presentation can be exacerbated by the additional defect in peroxisomal fission.

3.4.3 Mitochondria morphology in common neurodegenerative diseases: the case of Parkinson's Disease

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease. PD is characterized by the progressively diminished ability to initiate voluntary movement due to the loss of dopaminergic neurons in the *Substantia Nigra*. Most of the cases of PD are sporadic, but genetic studies identified pathogenic mutations in five genes: alpha synuclein, PTEN-induced putative kinase 1 (PINK-1), the cytosolic ubiquitin E3 ligase protein Parkin (Shimura et al., 2000), DJ-1 and leucine-rich repeat kinase 2 (LRRK2) (Lesage et al., 2009). A mitochondrial involvement in PD pathogenesis is strongly supported by evidence that in sporadic cases activity of the mitochondrial respiratory chain complex I is significantly impaired and that several of the proteins mutated in the familiar forms are localized on mitochondria and regulate their functions (Henchcliffe et al., 2008). Parkinson-like clinical syndrome can be induced by pharmacological inhibition of complex I and this treatment causes DRP1-dependent mitochondrial fragmentation (De Vos et al., 2005), suggesting a role for abnormal mitochondrial dynamics in the disease. Interestingly, prevention of mitochondrial fission by overexpression of Mfn1 or dominant negative DRP1 protects from neuronal cell death (Barsoum et al., 2006). Genetic factors associated with PD also cause changes in mitochondrial dynamics. The ablation of the sensor of oxidative stress DJ-1 in MEFs causes mitochondrial fragmentation (Blackinton et al., 2009). More controversial is the effect of the PINK1/Parkin pathway on mitochondrial shape. Studies in Drosophila indicate that ablation of PINK1 causes deep remodelling of cristae structure and the phenotype can be rescued by Parkin overexpression (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Moreover, modulation of fission and fusion machineries in Drosophila single knock out for PINK1 and Parkin suggests that this pathway promotes mitochondrial fission and/or inhibit fusion ((Deng et al., 2008; Poole et al., 2008; Park et al., 2009a; Yang et al., 2008). While some studies in mammalian cells support this notion (Yang et al., 2008;

Mortiboys et al., 2008), others show that silencing of PINK1 and Parkin cause mitochondrial fragmentation (Park et al., 2009b; Lutz et al., 2009; Exner et al., 2007; Sandebring et al., 2009), as also observed in fibroblasts from PD patients carrying PINK1 mutation (Exner et al., 2007; Grunewald et al., 2009). The role of parkin in mitochondrial dynamics may go beyond regulating fission and fusion. A recent study from Narendra and colleagues (2008) shows that parkin is recruited to mitochondria upon mitochondrial depolarization and promotes organelle fission and mitophagy (Narendra et al., 2008). Therefore, it is tempting to speculate that PINK1 and parkin work in tandem to coordinate fission of dysfunctional mitochondria and target them to degradation (Dagda and Chu, 2009; Cherra, III et al., 2009).

When this Thesis started, less was known about the role of mitochondrial dynamics in Huntington's Disease. Therefore, we focused on the study of mitochondrial morphological and structural alterations in HD and their contribution to the progression of the disorder. Molecular and pathological mechanisms involved in the pathogenesis of the disease will be described in detail in the following sections.

3.5 Huntington's Disease

Huntington's Disease is an autosomal dominant neurodegenerative disorder that occurs with a frequency of approximately 1 in 10,000 individuals in most populations of Caucasian descent. (Hayden et al., 1981; Harper, 1991; Vonsattel and DiFiglia, 1998). The earliest mentions of Huntington's Disease date back to the first half of the XIX century when symptoms, progression and heredity of the disease started to be described. The name of the disorder is given by the American physician George Huntington who in 1872 presented a detailed definition of the disease and unknowingly described its autosomal dominant inheritance pattern. The precise causal gene, already mapped in human chromosome 4 in 1983, was isolated 10 years later and the mutation responsible for the onset of the disease was described (1993). Although in the last years considerable advances have been done in the understanding of the pathophysiology of this devastating disorder, the pathological mechanisms underlying the syndrome haven't been completely unravelled and an effective therapy is still missing, making Huntington's Disease the subject of intensive and increasing research.

3.5.1 Genetics of Huntington's disease

Huntington's disease is an autosomal dominant genetic disorder caused by the expansion mutation beyond 36 CAG repeats in the IT15 gene, located in the short arm of chromosome 4 (4p16.3) (1993; Kremer et al., 1994). The mutation resides in the first exon of the gene encoding for the protein Huntingtin and results in the expansion of a polyglutamine domain.

The age of onset of symptoms inversely correlates with the CAG repeat number (1993; Duyao et al., 1993; Andresen et al., 2007) and expansions of approximately 70 glutamines are more present in juvenile case of HD. Homozygous patients show, despite a similar age of onset to heterozygous patient, higher severity and faster progression of the disorder (Squitieri et al., 2003). Moreover, particularly when the mutation is inherited paternally, the age of onset of the disease decreases due to an increase in the number of repeats in successive generations, a phenomenon termed genetic anticipation (Duyao et al., 1993; Leeflang et al., 1999; Wheeler et al., 1999; Kennedy and Shelbourne, 2000). Although the number of CAG repeats accounts in large part for the variance in the age of onset of the first symptoms, several studies show that heritable factors in humans can alter the course of HD. Thus, nowadays many research groups are focusing their efforts on the discovery of genetic modifiers that could disclose still unknown mechanisms and could also represent new potential therapeutic targets (Gusella and Macdonald, 2009).

3.5.2 Symptoms and clinical features

Huntington's Disease is associated with abnormality in voluntary and involuntary movements (choreoathetosis) that affects primarily limbs, the oro-bucco-facial regions and eyes and it progressively worsen over the course of the disease. Psychiatric disturbances like depression, apathy, violent behaviour are prevalent and may appear before the onset of significant motor impairment. Patients are commonly affected by cognitive deficits as impairment of general intellectual abilities and memory that are exacerbated in the late stages of the disease producing an intellectual state that approaches the range of mental retardation [for a review (Purdon et al., 1994)]. Moreover, patients shows muscle wasting and weight loss, despite constant caloric intake ((Sanberg et al., 1981; Kirkwood et al., 2001; Djousse et al., 2002a).The

majority of patients start to show the symptoms at 40-50 years of age and the disease terminates into death 10-20 years after the onset of the first clinical alterations (Martin and Gusella, 1986; Ho et al., 2001).

Huntington's disease is characterized by progressive atrophy, loss of neurons and gliosis mainly in the basal ganglia and secondarily in the cortex. The most affected tissue is the striatum (caudate and putamen nuclei) that shows massive loss of GABAergic projection medium-sized spiny neurons, that correspond to the 95% of the striatal neuronal population (Vonsattel et al., 1985; Ferrante et al., 1991). In late stages of the disease also non striatal structures are affected, as cortex, globus pallidus, thalamus,substantia nigra and cerebellum.

3.5.3 Huntingtin protein

Huntingtin is a 350kDa protein that bears no homology to any known protein. It is ubiquitously expressed with highest levels in brain, testis and to a lesser extent in heart, liver and lung (Schilling et al., 1995; Sharp et al., 1995). At the cellular level, the protein is found in the cytoplasm, in neuritis and synapses where it associates with various vesicular structures, endoplasmic reticulum, the Golgi apparatus and microtubules (DiFiglia et al., 1995; Gutekunst et al., 1995; Velier et al., 1998; Metzler et al., 2001). Huntingtin is also detected in the nucleus where it interacts with transcription factors and spliceosome related proteins (De Rooij et al., 1996; Kegel et al., 2002; Takano and Gusella, 2002; Zuccato et al., 2001a). Interestingly, the protein has been observed directly associated with mitochondria (Gutekunst et al., 1998; Panov et al., 2002a; Choo et al., 2004a). According to its subcellular localization, Huntingting exerts different functions, from the regulation of organelle trafficking and vesicular endocytosis in the cytoplasm, to the control of the expression of specific target genes in the nucleus. A strong contribution to the understanding of the physiological functions of Huntingtin comes from the identification of its numerous molecular interactors (Kaltenbach et al., 2007). Relevant examples include the Huntingtin associated protein 1 (HAP1) which interacts with the dynactin complex supporting a role for Huntingtin in intracellular transport (Li and Li, 2005; Li et al., 1998a) and huntingtin interacting protein 1 (HIP1) that binds alpha-adaptin and clathrin suggesting an involvement in endocytosis (Li et al., 1995; Kalchman et al., 1997; Wanker et al., 1997).

The generation of a KO mouse model for Huntingtin revealed that the protein is essential for embryonic development and it exerts an indispensable anti-apoptotic function since the null embryos show massive cell death in the ectoderm at E7.5 (Zeitlin et al., 1995a). Interestingly, embryonic lethality can be rescued by the expression of a mutant form of Huntingtin, suggesting that the protein function during development is independent from the length of the polyglutamine domain (Dragatsis et al., 2000). Huntingtin is also necessary for neurogenesis and normal hematopoiesis (White et al., 1997a; Metzler et al., 2000).

3.5.4 Models for Huntington's Disease

Several models for HD have been generated expressing the mutant gene in a number of different organisms, including C. Elegans, drosophila, mice and rats (Steffan et al., 2001; Faber et al., 1999; von Horsten et al., 2003). Rodent models appear to be the most useful for examining the contribution of different cellular mechanisms of disease and for testing the efficacy of putative therapeutics. Mouse models of HD fall into three broad categories:

(i)   mice expressing exon-1 fragments of human huntingtin gene containing polyglutamine mutations, in addition to both murine wt huntingtin alleles [R6/2 (Cha et al., 1998; Lodi et al., 2000); R6/1; N171 (Schilling et al., 1999); HD 100 and HD 46 (Laforet et al., 2001); Tet-Off (Yamamoto et al., 2000)];

(ii)   mice expressing the full length human huntingtin gene [YAC (Hodgson et al., 1999a); HD 89 (Schilling et al., 1999)];

(iii) mice with pathogenic CAG repeats inserted in the endogenous murine allele [Hdh knock-in (Wheeler et al., 2000; White et al., 1997b); CAG knock-in (Levine et al., 1999); Hdh 150Q (Lin et al., 2001) ; Hdh 4/6 knock-in (Shelbourne et al., 1999); HD/Hdh chimera (ishiguru H. 2001)].

Even if the phenotypes vary greatly between mouse models some generalities can be drawn from comparing the available mouse lines. Firstly, mice require longer CAG repeats than human to elicit pathogenic events. Second, the age of onset of the symptoms decreases when the N-terminal fragment of mutant huntingtin is expressed. However, although the expression of full length mutant huntigtin causes slower progression of the disease, the pattern of neurodegeneration resembles more closely human HD pathology.

Cell lines from peripheral tissues from HD patients have also been established. Even if the most affected tissue in HD is the striatum, myoblasts, fibroblasts and blood cell lines have been demonstrated to reflect changes observed in HD brains, hence representing a good model to study the disease. In particular, B lymphocytes cell lines have been widely used to study calcium homeostasis alteration and mitochondrial dysfunctions (Panov et al., 2002b; Almeida et al., 2008).

3.5.5 Pathological mechanisms

The mechanisms that lead to neuronal cell loss in Huntington's Disease are believed to be activated by the contribution of both the gain of toxic functions of mutant huntingtin and the loss of functions of the wt protein. It is worthy to mention that Huntington's Disease is one of the nine known inherited neurological diseases caused by the CAG repeat expansion mutation. This group of related diseases includes the spinal bulbar muscular atrophy (SBMA), several spinocerebellar ataxias (SCAs) and dentatorubral-pallidoluysian atrophy (DRPLA), each of them showing a different neurodegenerative pattern (Zoghbi and Orr, 2000). This suggests that although the mutation can evoke toxicity per se, the context of the protein in which it is present confers stringent mechanistic and tissue specificity.

In this section we summarize the main processes responsible for the deregulation of relevant intracellular pathways in HD.

3.5.5.1 Proteolytic cleavage of mutant huntingtin

A key step necessary to induce neurodegeneration in HD is the proteolytic cleavage of mutant Huntingtin (Wellington et al., 2000; Peters et al., 1999). The short forms of the protein containing a pathological polyQ expansion show higher toxicity than the full length with identical number of repeats. The short forms are found in the brain of patients and some murine models and they particularly accumulate in the nucleus (Hackam et al., 1998). Several proteases are responsible of the processing including caspase-1, -3, -6, calpains and yet uncharacterized aspartyl proteases (Kim et al., 2001; Graham et al., 2006). Consistently, inhibition of caspase cleavage by generation of a caspase-6-resistant form of mutant Huntingtin protected transgenic mice from striatal atrophy and neurodegeneration (Graham et al., 2006).

3.5.5.2 Aggregate formation: a toxic or protective event?

Proteins that carry an expanded polyglutamine domain often undergo conformational transition and are prone to aggregation (Scherzinger et al., 1997; Nagai et al., 2000). One of the major pathological hallmark of HD and other polyglutamine disorders is the presence of aggregates of the mutated protein both in the nucleus as neuronal intranuclear inclusions and in other cellular compartments such as neurites (DiFiglia et al., 1997a). Mutant Huntingtin has been proposed to form insoluble aggregates either through "polar zippers" or by transglutaminase-mediated events (Sipione and Cattaneo, 2001). The role of the nuclear inclusions in the progression of the disease is highly controversial since their formation doesn't correlate with neuronal cell death (Gutekunst et al., 1999a; Saudou et al., 1998; Kim et al., 1999; Arrasate et al., 2004); rather they could represent a protective mechanism of the cell to sequester and inactivate the soluble and more toxic pool of polyQ Huntingtin (Saudou et al., 1998). Although nuclear inclusions are not directly linked to induction of apoptosis they do cause the disruption of intracellular homeostasis and the development of neuronal dysfunction and severe symptoms, mainly sequestering the wt huntingtin molecules and other important cellular proteins. On the other hand neuropil aggregates correlate better with neuronal degeneration (Gutekunst et al., 1999b; Li et al., 2000).

3.5.5.3 Clearance of mutant huntingtin: inhibition of the proteasome and induction of autophagy

The brain seems uniquely susceptible to protein misfolding, as most of the major neurodegenerative diseases are associated with large intracellular inclusions (Zhou et al., 2003). Indeed, the mechanisms of protein quality control, degradation and clearance are altered in HD and the molecular regulators of these pathways are considered promising therapeutic targets. Mutant Huntingtin and intracellular inclusions are associated with proteasome impairment that may arise both from the inability of the proteasome to fully digest soluble expanded polyglutamine protein, releasing toxic polyglutamine stretches (Bence et al., 2001; Jana et al., 2001a; Venkatraman et al., 2004; Holmberg et al., 2004), and from the sequestration into aggregates of proteasome subunits and ubiquitin conjugation enzymes ((Jana et al., 2001a). Consistent with a role for the proteasome in the clearance of the mutant protein, aggregates formed by expanded Huntingtin are decorated by ubiquitin (Bennett et al.,

2007; Rubinsztein, 2006a; Williams et al., 2008; Williams et al., 2008; Williams et al., 2008; Rubinsztein, 2006b; Berger et al., 2006). Additionally, Huntingtin aggregates may sequester important players of the protein quality control machinery as chaperones Hsp40 and Hsp70, preventing them from exerting their protective function and ultimately leading to intracellular accumulation of misfolded proteins (Wyttenbach et al., 2000; Cowan et al., 2003). Thus, it is not surprising that induction of autophagy, a process whereby cells can eliminate aggregation prone proteins, has been shown to be protective in cells, Drosophila and mouse models for HD (Ravikumar et al., 2002; Ravikumar et al., 2004) (Williams et al., 2008). Interestingly, an increase in the number of autophagosomes has been reported in brains from HD patients (Sapp et al., 1997) and in cellular and mouse models. The increased autophagic activity is the result of the sequestration of the autophagy inhibitor mTOR into the polyglutamine aggregates, further supporting the protective role of aggregates (Ravikumar et al., 2004).

3.5.5.4 Transcriptional deregulation

Several reports indicate that mutant huntingtin affects the transcription of many functional classes of genes [for a review see (Sugars and Rubinsztein, 2003)], and of particular importance is the decreased expression of BDNF (brain derived neutrophic factor), an essential factor for differentiation and maintenance of neurons in the brain (Zuccato et al., 2001b). The molecular mechanisms by which mutant huntingtin can alter gene transcription span from the sequestration of transcription factors into aggregates, as first observed for the CREB-binding protein (Steffan et al., 2000) (Nucifora, Jr. et al., 2001) to the inhibition of the Sp1/TAFII130 (regulates dopaminergic receptor expression) transcriptional pathways by the direct interaction with the soluble polyQ protein (Yu et al., 2002) (Li et al., 2002) . Moreover, the mutation in huntingtin decreases its interaction in the cytoplasm with REST/NRSF (repressor element-1 transcription factor/neuron restrictive silencer factor) leading to the accumulation of the repressor in the nucleus where it inhibits the transcription of target genes as BDNF (Zuccato et al., 2003). More recently, mutant Huntingtin has been shown to down-regulate specifically in medium spiny neurons the expression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha), a transcriptional coactivator that regulates several metabolic processes as mitochondrial biogenesis and respiration (Weydt et al., 2006) .

3.5.5.5 Alterations of intracellular trafficking

Alteration of intracellular trafficking of vesicles and organelles participates in the development of HD and it represents an example of the loss of physiological functions of huntingtin due to the expansion of the polyglutamine domain. Indeed, while wt huntingtin can regulate anterograde and retrograde transport interacting, together with the partner HAP1, with dynein and kynesis motor complexes, the mutant form of the protein binds more tightly to such complexes, causing the detachment of motor proteins from microtubules and the general reduction in the transport of important neuronal factors as BDNF (Gauthier et al., 2004; Li et al., 1998b; Gunawardena et al., 2003). Moreover, mutant huntingtin can form aggregates that accumulate in axonal processes and terminals. Here they can sequester soluble motor proteins titrating them from other cargoes and pathways (Szebenyi et al., 2003; Trushina et al., 2004). Aggregates can also act as physical road blocks in the narrow axons, leading to the accumulation of vesicles and organelles in swollen axonal projection (DiFiglia et al., 1997b; Sapp et al., 1999).

3.5.5.6 Excitotoxicity

The "excitotoxicity hypothesis" is one of the hypotheses that have been put forward to explain the specific sensitivity of medium spiny neurons of the striatum to degeneration in HD. Excitotoxicity is defined as cell death deriving from the toxic action of excitatory amino acids (LUCAS and NEWHOUSE, 1957; Rothman and Olney, 1995). In mammalian central nervous system the major excitatory neurotransmitter is glutamate (review in (Hebb, 1970), and prolonged exposure to it can cause excessive influx of ions, especially calcium, and water into the cell, leading to the activation of enzymes that degrade proteins, nucleic acids and membranes (reviewed in (Berliocchi et al., 2005). The striatum receives massive glutamate signalling from cortical afferents and it is widely accepted that aberrant activity of the post-synaptic ionotropic glutamate receptors NMDA plays a role in the pathogenesis of the disease. Consistently, studies in post-mortem brains from HD patients show a preferential loss of striatal neurons with high expression of NMDAR (Young et al., 1988) and treatment of animal models with NMDAR agonists replicate behavioural and neuropathological features of HD (Schwarcz et al., 1984; Sanberg et al., 1989; Hantraye et al., 1990). In several mouse

models, an increased release of glutamate from cortical afferents onto striatal spiny neurons has been observed in early symptomatic stages (Cepeda et al., 2003), concomitantly with an impairment in clearance of glutamate from the synaptic cleft caused by reduced expression of glutamate transporter (EAAT1/GLT1) in astrocytes (Arzberger et al., 1997; Behrens et al., 2002). Moreover, the observation that in a variety of HD models striatal neurons showed enhanced NMDA-evoked currents suggested a functional interaction between polyQ expansion in Huntingtin and NMDAR activity (Laforet et al., 2001; Zeron et al., 2002; Zeron et al., 2004a). It has been proposed that mutant Huntingtin could increase the amount of receptor (and stabilize it) at the plasma membrane enhancing the forward intracellular trafficking, physically binding to common interactors as PSD95 (Sun et al., 2001; Fan et al., 2009) and altering post-transcriptional modifications of NMDAR subunits (Song et al., 2003). In addition to the alterations at the synaptic and receptor levels, HD neurons show perturbed calcium homeostasis and thereby defects in the response to the downstream signalling pathway initiated by NMDAR activation (Hodgson et al., 1999b; Seong et al., 2005; Hansson et al., 2001). This alteration can be in large part assigned to the fact that mitochondria in HD have reduced calcium buffering capacity and are more sensitive to calcium-induced depolarization eventually leading to enhanced apoptosis (Panov et al., 2002b; Choo et al., 2004b). Thus, mitochondria play a critical role in the process of excitotoxicity bearing the power of deciding neuronal cell fate. Mitochondrial alterations in HD will be discussed more in detail in the following chapter (Figure 2).

3.6 Mitochondria in Huntington's Disease

3.6.1 Metabolic defects

HD patients exhibit well described metabolic defects ((Leenders et al., 1986; Jenkins et al., 1993a) that have been linked to mitochondrial dysfunction. Patients show weight loss despite adequate caloric intake in the early stages of the disease (Djousse et al., 2002b) and positron emission tomography (PET) revealed that striatal glucose uptake and consumption is reduced in gene carriers even years before the onset of the motor symptoms (Kuhl et al., 1982; Antonini et al., 1996). Defects in glycolysis have also

been suggested by findings that symptomatic HD patients have elevated lactate production in basal ganglia and cortex, detected by proton nuclear magnetic resonance (1H-NMR) imaging (Jenkins et al., 1993b). Accordingly, skeletal muscle from HD patients and mutation carriers show a significant delay in the recovery of phosphocreatine levels after exercise (Saft et al., 2005), indicative of a decreased synthesis of ATP.

A number of studies revealed impaired activity of enzymes of the tricarboxylic acid (TCA) cycle, in particular of aconitase (Tabrizi et al., 1999), and of the mitochondrial respiratory chain complexes II, III (Stahl and Swanson, 1974; Brennan, Jr. et al., 1985) and IV (Browne et al., 1997a) in HD striatum. The relevance of succinate dehydrogenase (SDH) defects to the HD phenotype is underscored by the fact that systemic administration of mitochondrial toxins that selectively inhibit SDH, namely 3-nitropropionic acid (3-NP) and malonate, induce striatal-selective lesions in humans and animal models that resemble those seen in HD (Browne and Beal, 2002).

Further evidence that energetic defects contribute to neurodegenerative process in HD is provided by the findings that agents that enhance energy production in the brain exert beneficial effects. Indeed, administration of coenzyme Q10 (Koroshetz et al., 1997; Ferrante et al., 2002), creatine (Matthews et al., 1998; Ferrante et al., 2000) or the pyruvate dehydrogenase activator dichloroacetate (Andreassen et al., 2001) to mouse models and patients slows the progression of the disease and attenuates striatal degeneration.

The molecular basis of the impaired mitochondrial energy production in HD remains unclear. Recently, a role for PGC-1alpha (peroxisome proliferator-activated receptor-gamma coactivator 1alpha), a transcription factor implicated in energy homeostasis, adaptive thermogenesis, beta-oxidation of fatty acids, and glucose metabolism regulation[for a review (Puigserver and Spiegelman, 2003)], has been proposed in HD. Striata from HD patients and mouse models show decreased expression levels of PGC1alpha and its target genes. How can mutant huntingtin influence PGC-1alpha levels and activity? Cui and colleagues (2006) suggest that mutant huntingtin may interfere with the transcription of PGC-1alpha, whereas Weydt and colleagues (2006) propose that mutant huntingtin may bind to PGC-1alpha, impairing its ability to upregulate expression of its downstream target genes. However, further studies are necessary to definitively demonstrate the role of PGC-1alpha in HD.

A direct consequence of the impaired ATP production is the disruption of cellular energy-dependent processes, as the control of resting membrane potential in neurons by the Na^+/K^+ ATPase pump. If the impairment of the ATPase pump is severe enough to prevent membrane repolarization, voltage dependent ion channels as NMDAR can be activated even in presence of normally inert levels of agonist, leading to excitotoxicity (Novelli et al., 1988; Zeevalk and Nicklas, 1991). The reduced production of ATP also results in decreased cellular cAMP levels and reduced cAMP responsive element (CRE)-mediated gene transcription (Gines et al., 2003). Of note, one of the target genes of this pathway is BDNF that shows reduced expression in HD. Furthermore, the adenylate cyclase stimulator forskolin abrogates toxicity induced by the expression of mutant huntingtin in neuronal PC12 cell line (Wyttenbach et al., 2001).

3.6.2 Apoptosis

The role of apoptosis in neurodegeneration associated with Huntington's Disease has been highly debated [review (Hickey and Chesselet, 2003)]. The increased activation of certain apoptotic pathways and the enhanced activity of caspases in HD are widely accepted to deeply contribute to the pathology, deregulating cellular homeostasis and promoting the proteolytic processing of mutant huntingtin further augmenting its toxicity. However the evidence for a pure apoptotic process contributing to HD cell death is controversial. Several studies show that striatal neurons from HD brains are characterized by enhanced cytochrome c release from mitochondria to the cytosol (Kiechle et al., 2002) and increased activity of calpain (Gafni and Ellerby, 2002), caspase-1 (Ona et al., 1999) and -8 (Sanchez et al., 1999). Early studies clearly identified apoptotic-like cells by TUNEL labelling on post-mortem striatum from HD patients (Dragunow et al., 1995a; Portera-Cailliau et al., 1995a; Thomas et al., 1995), but the apoptotic specific pattern of DNA laddering was not always confirmed, raising the possibility that other cell death processes as necrosis can occur in parallel with apoptosis (Dragunow et al., 1995b; Portera-Cailliau et al., 1995b). It is worth mentioning that the detection of apoptosis in patients brains can be influenced by the amount of delay before autopsy and more importantly by the fact that apoptosis is a transient event and affects only a limited number of cells at a time (Gavrieli et al., 1992). Furthermore, the observation of activated astrocytes in HD brains suggests

neuroinflammation, which is generally absent in apoptotic conditions (Hedreen and Folstein, 1995).

Genetic models for HD show different extent and timing in the appearance of apoptotic neurons in the striatum and this seems to depend on the specific rate of progression of the pathology and pattern of neurodegeneration that characterize each model (Turmaine et al., 2000; Iannicola et al., 2000; Reddy et al., 1998). Interestingly, neurochemical models, as that one induced by the administration of the 3-nitropropionic acid (3-NP), show a clear activation of the intrinsic apoptotic pathway (Almeida et al., 2004; Kim and Chan, 2001).

The link between apoptosis and mutant huntingtin has been clarified by many works on in vitro models. Expression of full length or N-terminal fragments of mutant huntingtin in different neuronal and non neuronal cell lines have been shown to induce cytochrome c release, caspase activation and DNA fragmentation in a CAG repeat number-dependent fashion (Jana et al., 2001b; Liu, 1998). Striatal cell lines isolated from different mouse models show higher rates of apoptosis upon NMDAR activation. Moreover, lymphoblasts and myoblasts from HD patients display increased sensitivity to mitochondria-mediated apoptosis and the pan-caspase inhibitor zVAD can protect from cell death (Sawa et al., 1999; Ciammola et al., 2006). Importantly, p53, a transcription factor that regulates the expression of various proteins, such as the proapoptotic proteins Bax and Puma (Miyashita and Reed, 1995), is shown to interact with the N-terminal part of mutant huntingtin in HD lymphoblasts (Steffan et al., 2000) and neuronal cell lines expressing the mutant protein. This interaction leads to increased nuclear levels and transcriptional activity of p53, phenomenon observed also in HD mice and patients. Interestingly the genetic ablation and the inhibition of p53 suppresses neurodegeneration in different models for HD (Bae et al., 2005).

A further support for a role of the activation of apoptosis in HD is the evidence that wt huntingtin exerts an anti-apoptotic effect and this function is lost upon mutation. As mention above, ko mice for Huntingtin are embryonically lethal and show massive apoptotic cell death at E7.5 (Zeitlin et al., 1995b). Moreover, a work from Rigamonti R. (2000) and colleagues shows that expression of wt huntingtin in neurons protects from different stresses as proapoptotic stimuli. The authors show that huntingtin acts upstream of caspase-3 activation, inhibiting the conversion of procaspase-9 into active caspase-9.

Another mechanisms whereby wt huntingtin exerts its antiapoptotic effect may involve the interaction with HIP-1. When the polyQ domain is expanded, the huntingtin/HIP-1 interaction is disrupted and HIP-1 is released. HIP-1, which bears a death effector domain, can then bind to its partner hippi that in turn activates caspase-8 leading to apoptosis (Gervais et al., 2002).

Interestingly, minocycline, a tetracycline analog, that has been reported to exert neuroprotective effect over various experimental models, slows the progression of symptoms as psychiatric and motor disturbances in patients and mouse models for HD. Besides its anti-inflammatory effect, minocycline is also known to reduce mitochondrial permeability transition mediated cytochrome c release and the expression of caspase-1 and -3 (Bonelli et al., 2003). More recently, a screening identified a compound, methazolamide, that inhibits cytochrome c release, likely acting at the level of outer mitochondrial permeabilization, and is effective in slowing the progression of HD in R6/2 mice (Wang et al., 2008).

3.6.3 Oxidative stress

The generation of reactive oxygen species (ROS) and the resulting oxidative stress is a feature of Huntington's Disease (Browne et al., 1997b; Grunewald and Beal, 1999). Studies of post-mortem human HD brains show high levels of oxidative damage as cytoplasmic accumulation of lipofuscin, a product of unsaturated fatty acids peroxidation (Braak and Braak, 1992), DNA strand breaks (Dragunow et al., 1995b) and oxidative markers in mitochondrial and nuclear DNA bases (Polidori et al., 1999), and protein nitration (Browne et al., 1999). Moreover, the oxidative alterations observed in patients are recapitulated in genetic and pharmacological animal models for HD [for a review see (Browne and Beal, 2006; Tolkovsky et al., 2002)]. Mitochondria are considered the primary source of ROS in neurons and mitochondrial dysfunctions in HD, such as ATP production defects due to inhibition of electron transport chain and abnormal calcium handling especially during exposure to excitotoxins, are likely to be linked to the increase in ROS levels. However, a recent work from Brennan and colleagues (2009) shows that neurons mainly rely on NADPH oxidases (NOX) to produce ROS during excitotoxicity, leading us to re-examine the role of mitochondria in neuronal cell death.
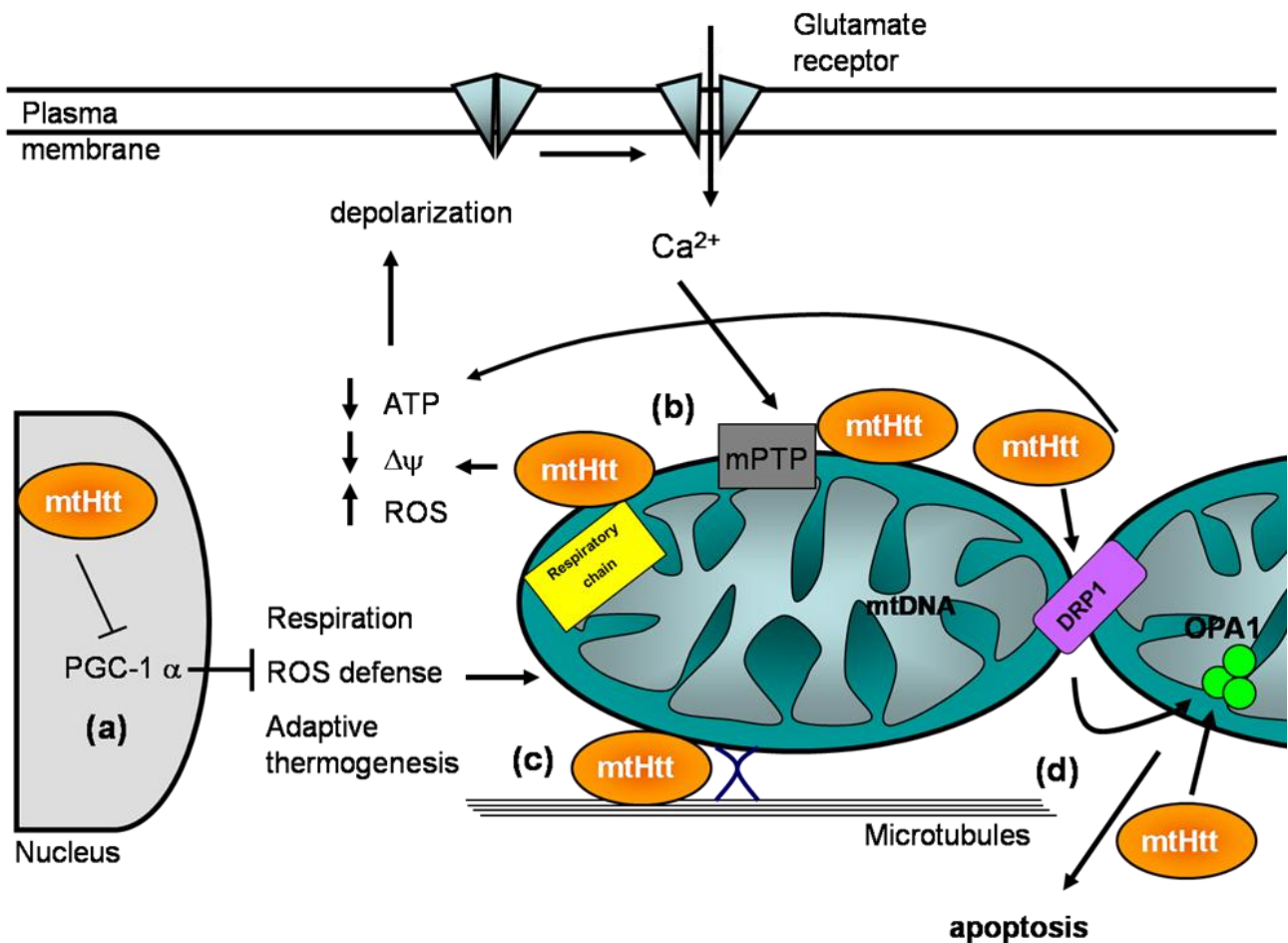
Figure 2. Mitochondrial dysfunctions in Huntington's Disease. (**a**) mHtt block of PGC1-α promoter results in decreased expression of nuclear-encoded mitochondrial proteins that are necessary for respiration and oxidative damage defense. (**b**) mHtt can have direct effects on mitochondria inhibiting complex II and increasing sensitivity to Ca^{2+}-induced mitochondrial permeability transition pore (mPTP) opening. Respiratory inhibition leads to decreased energy production and mitochondrial membrane potential ($\Delta\psi_m$) in addition to increased reactive oxygen species (ROS) production. The bioenergetic decline can cause increased vulnerability to excitotoxic stimuli. (c) mHtt interferes with mitochondrial trafficking along microtubules directly interacting with motor complexes. (d) mHtt alters mitochondrial morphology and cristae structure.

3.6.4 Deregulation of calcium homeostasis

Strong evidence support a role of mitochondrial regulation of Ca^{2+} homeostasis in HD. Several studies on mitochondria from HD patients lymphoblasts, mouse models brains and neuronal HD cell lines revealed a reduction in mitochondrial membrane potential and an enhanced sensitivity to Ca^{2+}-induced permeability transition (Panov et al., 2002b; Choo et al., 2004b; Gizatullina et al., 2006). A key study in this field from Panov and colleagues (2002) demonstrated that the mitochondrial calcium retention capacity reduction correlates with the length of the polyglutamine expansion in Huntingtin. Moreover, the authors showed that the incubation of isolated mitochondria with GST

fusion proteins containing expanded polyglutamine domains can directly reduce mitochondrial membrane potential and impair Ca^{2+} handling. Consistently, isolated mitochondria from mouse liver show decreased calcium threshold for mPTP opening if incubated with a recombinant N-terminal fragment of Huntingtin bearing an expanded polyglutamine domain (Choo et al., 2004b). These findings support the hypothesis that mutant huntingtin can cause mitochondrial dysfunctions through the physical interaction with this organelle.

Mitochondria from brain from YAC72 HD mice show slower clearance of extra-mitochondria calcium and inability to return to baseline calcium levels (Panov et al., 2002b). At the cellular level these dysfunctions result in slower recovery rate of cytosolic calcium concentration upon NMDAR activation and increased mitochondrial depolarization upon sustained NMDAR stimulation (Fernandes et al., 2007), ultimately leading to permeability transition dependent cytochrome c release and apoptosis (Zeron et al., 2004b).

3.6.5 Alterations in mitochondrial movement

As we discussed above, mitochondria are not only the main source of energy of the cell but also vital regulators of calcium homeostasis. To properly exert their functions, mitochondria must be trafficked appropriately to reach specific cellular compartments with high energy and calcium buffering demands, such as synapses. This has a specific relevance in neurons where axons and dendrites can extend well beyond the cell body. In addition, mitochondrial trafficking may be important for transporting damaged mitochondria to cellular locations where they can be repaired or degraded (De Vos et al., 2000). Indeed, the persistence of injured mitochondria can lead to increased reactive oxygen species production and apoptosis (Lee and Wei, 2000; Orr et al., 2008a).

Several studies in neurons from HD mouse models show an impairment in the trafficking of mitochondria along axons. HD mitochondria show abnormal distribution in the cell, they travel shorter distances than their normal counterparts and they appear to stop more frequently becoming progressively immobilized (Trushina et al., 2004) (Orr et al., 2008a; Mormone et al., 2006). Different mechanisms have been proposed to explain this phenotype. Trushina and colleagues (2004) showed that in mouse and HD patients neurons mutant Huntingtin aggregates sequester wt huntingtin and the soluble

pool of motor proteins leading to a general decrease in mitochondrial trafficking. On the other hand, the work of Chang and colleagues (2006) showed that mitochondrial movement is specifically decreased at the sites of cytosolic mutant huntingtin aggregates that act as physical roadblocks in the narrow axons. A further hypothesis is that the association of the N-t fragment of mutant huntingtin with mitochondria can reduce the interaction of the organelles with microtubule-based transport proteins, leading to the trafficking phenotype (Orr et al., 2008b). It would be interesting to elucidate the mechanisms causing the defects in mitochondrial trafficking since they seem to appear earlier than other mitochondrial alterations, suggesting that they can contribute to mitochondrial dysfunction.

3.6.6 Mitochondrial morphology and ultrastructure

Mitochondria are dynamic organelles that undergo morphological and ultrastructural changes in respose to specific physiological and pathological conditions. A growing body of evidence shows that mitochondrial morphology intimately correlates with mitochondrial functional parameters as ATP production, ROS generation, movement and apoptosis. In HD, early studies on brain biopsies from patients revealed the presence of mitochondria characterized by abnormal ultrastructure and morphology (Tellez-Nagel et al., 1974; Goebel et al., 1978). Alteration in mitochondrial cristae structure has been observed also in excitotoxic models for HD (Portera-Cailliau et al., 1995b). Despite the early observation of these alterations, it is currently unknown which is the role of mitochondrial structural defects in the progression of the disease and which is the mechanism whereby mutant huntingtin can influence mitochondrial morphology. Indeed, in the last few years many groups have started to focus their research on this previously unexplored topic. Electron microscopy analysis on lymphoblasts, myoblasts and fibroblasts from HD patients showed that mitochondria display less and remodelled cristae and the severity of ultrastructural defects correlates with the genotype (Squitieri et al., 2006; Squitieri et al., 2009). Moreover, in lymphoblasts from heterozygous patients mitochondria appear redistributed and clustered at one pole of the cell (Mormone et al., 2006), suggesting that alterations occur not only at the ultrastructural level but the pattern of the mitochondrial network is also affected. A recent work from Wang and colleagues (2008) shows that expression of the N-terminal fragment of mutant Huntingtin in HeLa cells induces reduction in the

movement and in the fusion rate of mitochondria. Additionally, upon serum starvation or H_2O_2 treatment cells bearing mutant Huntingtin display deep fragmentation of the mitochondrial network, reduction in ATP levels and enhanced cell death. Interestingly, these defects can be corrected both inhibiting the fission machinery overexpressing a dominant negative form of Drp1 and promoting mitochondrial fusion through the overexpression of Mfn2 (Wang et al., 2009a). Fragmentation of the mitochondrial network has been observed also in a pharmacological model for HD. Primary cortical neurons treated with 3-NP show an immediate drop in ATP levels and a mild rise in reactive oxygen species followed by a NMDA activation-dependent more pronounced rise in ROS that ultimately causes mitochondrial fragmentation and neuronal cell death (Liot et al., 2009).

These findings suggest that mitochondria shape changes play a role in neuronal cell death and that the correction of morphological defects can potentially restore metabolic and other mitochondrial functional alterations associated with HD. However, although the description of the morphological defects in HD is becoming more detailed and complete, the mechanism that links Huntingtin mutation to mitochondria shape and the responsible molecular players are still unknown.

The aim of this thesis project was therefore to understand

(i)   which pathways impinging on mitochondrial morphology and ultrastructure are altered in HD,

(ii)   how they affect mitochondrial morphology, cell dysfunction and death.

(iii)   Whether they can be targeted to correct the viability of cells from HD mode

4 Results

Cereghetti GM, Costa V, Scorrano L.
Inhibition of Drp1-dependent mitochondrial fragmentation and apoptosis by a polypeptide antagonist of calcineruin.
Cell Death and Differentiation, in revision.

Costa V, Lim D, Carafoli E, Malorni V, Scorrano L.
Mitochondrial fission and cristae disruption sustain apoptosis in cellular models of Huntington's disease.
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Inhibition of Drp1-dependent mitochondrial fragmentation and apoptosis by a polypeptide antagonist of calcineurin.

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Running Title: PPD1, calcineurin and Drp1 in apoptosis

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Abstract

During apoptosis mitochondria lose their membrane potential and undergo fragmentation around the time of release of cytochrome *c*. Apoptotic fission is at least in part sustained by the translocation of dynamin related protein 1 (Drp1), normally located in the cytosol, to mitochondria. This process depends on dephosphorylation of Drp1 by the phosphatase calcineurin. Here we report the identification of a novel inhibitor of this process. A polypeptide (PPD1) from the immunophilin FKBP52, inhibits calcineurin activation triggered by mitochondrial dysfunction. PPD1 blocks Drp1 translocation to mitochondria and fragmentation of the organelle. PPD1 delays apoptosis by intrinsic stimuli, by preventing fragmentation and release of cytochrome *c*. Cells expressing PPD1 display enhanced clonogenic ability following exposure to staurosporine. A genetic analysis revealed that the activity of PPD1 is independent of the BH3-only protein BAD, another target of calcineurin during apoptosis, and is not additive to inhibition of Drp1. Thus, PPD1 is a novel inhibitor of apoptosis that elucidates the role of calcineurin-dependent mitochondrial fragmentation in the amplification of cell death.

Introduction

Mitochondria are key organelles in the regulation of apoptosis. They amplify the intrinsic pathway by releasing protein cofactors that are essential for the activation of effector caspases (like cytochrome *c*) and for the direct condensation of chromatin (like AIF) (1). This release is the consequence of permeabilization of the outer membrane of the organelle and it is controlled by members of the Bcl-2 family. In a widely accepted model, the subset of "BH3-only" Bcl-2 family members senses the "private" death stimuli, transferring it to mitochondria whose outer membrane is permeabilized via the "multidomain" proapoptotic members. The action of BH3-only proteins is counteracted by antiapoptotic members that prevent activation of the multidomains in the mitochondrial membrane, by soaking the BH3-only members (2, 3). Thus, in this model the only mitochondrial event that plays a key role in the progression of the apoptotic cascade is loss of the controlled permeability of the outer membrane of the organelle. One of the basic tenets of apoptosis, that mitochondrial shape is unchanged, has been recently challenged thanks to the development of more refined techniques of investigation of mitochondrial shape and ultrastructure. A new consensus developed that mitochondria undergo massive fragmentation around the time of their outer membrane permeabilization (4, 5), and that the complete release of cytochrome *c* is accompanied by ultrastructural changes collectively called cristae remodelling (6).

Mitochondrial shape is determined by the equilibrium between continuously ongoing fusion and fission processes. In mammalian cells, a core machinery of "mitochondria-shaping" proteins exists that impinges on fusion or fission of the organelle. Mitochondrial fusion depends on the outer membrane mitofusins (Mfn) 1 and 2 and on the inner membrane Optic Atrophy 1 (Opa1) (7). These proteins are large dynamin related GTPases that display pleiotropic functions: for example, Opa1 regulates biogenesis and remodeling of the cristae independently of its role in fusion (8, 9) while Mfn2 tethers mitochondria to the

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endoplasmic reticulum (10). Mitochondrial fission depends on the translocation of cytoplasmic dynamin related protein 1 (Drp1) to mitochondria, where it binds to its adaptor in the outer membrane, Fis1, oligomerizes and constricts the organelle, ultimately leading to its fission (11, 12). Translocation of Drp1 to mitochondria depends on its dephosphorylation by calcineurin and other phosphatases (13, 14),

The molecular knowledge of the players in mitochondrial shape regulation stimulated intense research in the last few years on their role in cell death. In particular, a dominant negative mutant of Drp1, in which substitution of lysine 38 for an alanine in the GTPase domain disrupted its enzymatic activity, prevents fragmentation of the mitochondrial network, mitochondrial membrane depolarization, cytochrome c release and cell death induced by staurosporine, placing Drp1 and mitochondrial fragmentation early in apoptosis (5). Further, Drp1 was found to accumulate at mitochondrial fission sites together with Bax and Mfn2 (15). Dominant negative Drp1 was shown not to impede Bax translocation to mitochondria, implying that Bax translocation alone is not sufficient for cell death induction (15-18). Interestingly, downregulation of Drp1 by shRNA showed that even if inhibiting cytochrome c release, absence of Drp1 was not sufficient to prevent release of other proapoptotic factors from mitochondria upon induction of cell death, suggesting that mitochondrial subcompartimentalization of cytochrome c stores might be altered by Drp1 (19, 20). Accordingly, remodeling of the cristae can be also blocked by a dominant negative mutant of Drp1 during apoptosis induced by the BH3 only protein BIK (21). Recently, Nunnari and colleagues found that mdivi-1, a specific chemical inhibitor of Drp1 GTPase activity, prevents both mitochondrial fission and Bax-mediated mitochondrial outer membrane permeabilization (22). While there is mounting evidence that mitochondrial fission participates in apoptosis, recent reports questioned whether genetic maneuvers aimed at upregulating mitochondrial fusion are cytoprotective (23), leaving the question of whether mitochondrial fragmentation is a decision step towards cell death.

We recently showed that Drp1 interacts with the cytoplasmic peptidylprolyl isomerase cyclophilin A in the cytosol. This interaction is lost upon induction of mitochondrial fragmentation triggered by depolarization of the organelle and calcineurin activation (24). Upon dephosphorylation of Drp1 at the Ser 637 residue by calcineurin Drp1 translocates to mitochondria causing their fission. This calcineurin loop in principle could provide a target to manipulate apoptotic mitochondrial fission. However, two problems should be taken into account when addressing this possibility. First, compounds that inhibit calcineurin, like cyclosporine A (CsA), also act on the permeability transition pore (PTP), whose role in at least certain paradigms of cell death is well established. Second, it has been shown that cyclophilin A is associated with dynamitin, a component of the dynein associated dynactin molecular motor complex responsible for retrograde protein transport along the microtubules network, and this association can be blocked by CsA (25). Dynein has also been implied in the translocation of Drp1 to mitochondria, thus complicating the picture (28). This raises the question of whether inhibitors of calcineurin affect translocation of Drp1 to mitochondria specifically by acting on dephosphoprylation of Drp1, or whether it acts via an indirect action mediated by dynein. In an attempt to find an answer to these questions, our attention was caught by a peptide corresponding to the peptidylprolyl isomerase domain of immunophilin FK506 binding protein 52 (FKBP52; residues ranging from glycine 32 to lysine 138), which is able to inhibit association between cyclophilin A and dynein (25, 27). This peptide represents a useful tool to address these questions. Here we show that expression of the FKBP52 peptide (PPD1) prevents Drp1 interaction with cyclophilin A, but does not dissociate Drp1 from dynein. PPD1 still inhibits mitochondrial fragmentation by blocking calcineurin activity and preventing recruitment of Drp1 to mitochondria. PPD1 prevents mitochondrial fragmentation, cytochrome c release and cell death induced by different apoptotic stimuli. Its action is independent of Bad, another important target of calcineurin during apoptosis, and is not additive to blockage of

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Drp1. Thus, blockage of calcineurin by PPD1 further supports a role for mitochondrial fragmentation during apoptosis and it unveils a novel potential site of action to intervene in therapy of pathological conditions where excess apoptosis should be blocked.

Results

PPD1 inhibits depolarization induced mitochondrial fragmentation

Mitochondrial depolarization triggers calcineurin activation, dephosphorylation of Drp1 and its translocation to mitochondria, both blocked by the calcineurin inhibitors FK506 and cyclosporine A (CsA). In fact, Drp1 interacts with calcineurin and with cyclophilin A and it is largely associated to microtubules. Upon mitochondrial dysfunction, Drp1 leaves this complex and the microtubules translocating to mitochondria (28). Interestingly, the microtubule anchored dynein motor complex regulates Drp1 translocation to mitochondria (26) and CypA was shown to act as a mediator of binding of proteins to the dynein motor complex (25). Thus the inhibitory effect of FK506 and CsA might depend on their ability to displace Drp1 from dynein. To test this possibility, we turned to PPD1, a peptide corresponding to the peptidylprolyl isomerase domain of immunophilin FK506 binding protein 52 (aa. 32-138) that displaces CypA from dynein. We therefore checked whether PPD1 had any effect on mitochondrial depolarization or fragmentation. Expression of PPD1 did not prevent mitochondrial depolarization induced by the lipid arachidonic acid (ArA), as shown by real time imaging of the mitochondrial fluorescence of the potentiometric dye tetramethyl rhodamine methylester (TMRM) loaded in HeLa cells cotransfected with GFP and PPD1 or an empty vector (Figure 1a). Similarly, expression of PPD1 did not affect depolarization by the protonophore carbonylcyanide p-trifluoromethoxyphenyl-hydrazone (FCCP). ArA and FCCP induce rapid fragmentation of HeLa mitochondria, causing the transition from network to individual organelles in approximately 10 minutes. Of note, PPD1 greatly delayed depolarization-induced, Drp1-dependent mitochondrial fragmentation, even if it had no effect on mitochondrial depolarization (Figure 1b-*c*).

PPD1 displaces cyclophilin A from Drp1 and inhibits calcineurin activation

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PPD1 could therefore represent a novel inhibitor of mitochondrial fragmentation acting by displacing Drp1 from dynein. To test this hypothesis, we immunoprecipitated Drp1 in HeLa cells and verified how PPD1 and mitochondrial dysfunction modulated its association with dynein. Western blot analysis of the co-immunoprecipitated material showed that interaction of Drp1 with CypA was diminished by expression of PPD1 (Figure 2a). On the contrary, interaction of Drp1 with dynein was not influenced by the presence of the peptide, indicating that interaction of Drp1 with the dynein motor complex is not mediated by the immunophilin (Figure 1a). While association of Drp1 with cyclophilin A was completely lost upon induction of mitochondrial fragmentation by ArA, interaction of the protein with dynein persisted. Thus, modulation of binding to dynein could not account for the inhibitory effect of PPD1 on mitochondrial fragmentation. We therefore turned to the possibility that PPD1 influenced calcineurin activation. A specific assay of calcineurin activity indicated that following mitochondrial dysfunction by both ArA and FCCP activity of the phosphatase increases and that this is completely abrogated in cells expressing PPD1 (Figure 2b). Thus, PPD1 acts as a calcineurin inhibitor that is genetically encoded and therefore more stable as opposed to the small pharmacological inhibitors that are metabolized like CsA and FK506.

PPD1 inhibits cell death at the mitochondrial level

Since PPD1 displayed the ability to inhibit calcineurin activity, we verified if it also acted on the translocation of Drp1 to dysfunctional mitochondria. To this end we monitored mitochondrial association of a Drp1-YFP chimeric protein co-expressed in HeLa cells with a mitochondrially targeted dsRED (mtRFP). Following mitochondrial depolarization induced by ArA or FCCP, Drp1-YFP readily translocated to mitochondria and this process was inhibited by contransfection with PPD1 (not shown). Thus, PPD1 blocks association of Drp1 with dysfunctional mitochondria. This suggests that PPD1 could interfere with

apoptotic settings where the fragmentation of mitochondria is essential for the progression of the cascade. To this end, we tested whether expression of PPD1 delayed kinetic of apoptosis by a panoply of intrinsic inducers acting via different mechanisms. Expression of PPD1 was able to delay death by staurosporine, etoposide and thapsigargin, three intrinsic stimuli that recruit the mitochondrial pathway of apoptosis controlled by Bax and Bak at the mitochondrial level, as well as by the pro-oxidant H_2O_2 that recruits mitochondria via the so called "ER gateway" similarly controlled by the two multidomain proapoptotics (29), (Figure 3). Thus, PPD1 delays apoptosis by stimuli that recruit mitochondria, irrespective of their initial mechanism of action, provided that they all cause mitochondrial fragmentation (5, 9, 30). We further verified if this was associated with an inhibition of mitochondrial morphological changes induced by apoptotic stimulation. We therefore followed in real time the changes in mitochondrial shape in HeLa cells by imaging the fluorescence of expressed mtRFP. Upon treatment with staurosporine mitochondria underwent the previously reported cycle of initial elongation followed by fragmentation that becomes apparent 2 hours after induction of apoptosis. Co-expression of PPD1 with mtRFP prevented fragmentation induced by staurosporine (Figure 4a). We therefore turned to analyze whether PPD1 was also capable of inhibiting release of cytochrome *c* from mitochondria, by evaluating its subcellular distribution using an immunofluorescence and a semi-quantitative image analysis approach (31) and found that PPD1 prevents also the release of cytochrome *c* (Figure 4b-c). We finally set to test whether PPD1 just delayed death, or whether it exerted a real cytoprotective effect. We therefore measured the intrinsic ability of the survived cells to replicate and form colonies, an ultimate sign that cells have completely survived the apoptotic attack. To this end HeLa cells were co-trasfected with GFP and PPD1 (or its empty vector), sorted, plated and treated with staurosporine. After 5 hrs cells were collected and re-plated with fresh medium devoid of apoptotic inducer. A count of individual colonies produced by the re-plated cells showed

that cells expressing PPD1 were able to survive and generate new colonies despite the treatment with staurosporine (Figure 4d-e). Taken together, these data indicate that PPD1 is capable of preventing cell death induced by stimuli that trigger mitochondrial permeabilization.

PPD1 inhibits cell death via the Drp1 pathway

Calcineurin affects a plethora of key cellular processes. For example, it dephosphorylates the proapoptotic Bcl-family protein BAD to induce its migration from the cytosol to mitochondria, leading to cell death (32). Thus, we wished to ascertain whether PPD1 protects from cell death specifically by blocking the Drp1 pathway. To this end we first had to exclude that the effects of PPD1 were due to its ability to block dephosphorylation of BAD. We therefore turned to a genetic approach, comparing the ability of PPD1 to block apoptosis by staurosporine in wt and $Bad^{-/-}$ mouse embryonic fibroblasts (MEFs). As expected, apoptosis by staurosporine was reduced in $Bad^{-/-}$ MEFs as compared to their wt counterparts (Figure 5). Expression of PPD1 was able to protect from apoptosis induced by staurosporine also in MEFs, irrespective of the presence of BAD. Thus, BAD is dispensable for the antiapoptotic action of PPD1. We then set out to investigate if the effect of PPD1 was mediated by Drp1. To this end we verified if the effect of PPD1 was additive to that of blocking Drp1. Expression of a dominant negative Drp1 mutant (Drp1^{K38A}) was capable of delaying death by staurosporine, like PPD1. Of note, co-expression of PPD1 with Drp1^{K38A} did not result in an additional protection, indicating that they act via the same pathway (Figure 5). In conclusion, our data indicate that the inhibition granted by PPD1 is not mediated by BAD and depends on the calcineurin-Drp1 axis.

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Discussion

In this study, we show that inhibition of the calcineurin-Drp1 axis by the peptide PPD1, corresponding to the peptidylprolyl isomerase domain of FKBP52, inhibits apoptosis. This is supported by a number of findings: (i) expression of PPD1 inhibits activation of calcineurin following mitochondrial dysfunction; (ii) PPD1 does not detach Drp1 from dynein, ruling out a possible alternative mechanism of inhibition of fragmentation; (iii) PPD1 blocks mitochondrial fragmentation, release of cytochrome *c* and apoptosis by a number of intrinsic cell death stimuli, and promotes survival of cells treated with staurosporine; (iv) a genetic analysis reveals that inhibition of cell death by PPD1 does not require BAD, another target of calcineurin during apoptosis, and is not additive to inhibition of Drp1.

PPD1 abrogates interaction of Drp1 and cyclophilin A in HeLa cells, but it does not dissociate Drp1 from dynein, the main component of the microtubules motor complex. Indeed this peptide prevents association of immunophilin FKBP52 with dynein (25); the ability of the peptide to also blunt binding of another immunophilin, cyclophilin A, to Drp1 suggests that the interaction between the immunophilin and Drp1 involves formation of a ternary complex between Drp1, cyclophilin A and dynein. In addition, Drp1 association with the dynein motor complex is not disrupted by induction of mitochondrial fragmentation with ArA, supporting a role for the dynein motor complex as the carrier of Drp1 to mitochondria as previously suggested (26). The key proapoptotic molecule Bim is also associated with the dynein motor complex and this association is lost during induction of apoptosis (33), suggesting scenarios of interplay between the apoptotic and the mitochondria-shaping machineries. Our data indicate that PPD1 inhibits calcineurin. Since Drp1 interacts with both cyclophilin A and calcineurin (28), we could figure out that the ability of the peptide to inhibit calcineurin is reinforced by the fact that the proteins meet on microtubules and calcineurin is sequestered and inactivated by the cyclophilin A-PPD1 complex.

PPD1 is an efficient inhibitor of mitochondrial fragmentation induced by mitochondrial depolarization. It also blocks fragmentation induced by apoptotic stimuli, like staurosporine. Even if the role of mitochondrial fragmentation in cell death remains controversial, this finding prompted us to extend our analysis to investigate if PPD1 displayed any anti-apoptotic potential. Interestingly, PPD1 prevents release of cytochrome c induced by staurosporine and delays apoptosis by a number of intrinsic stimuli that trigger death by different mechanisms (staurosporine, etoposide, H_2O_2 and thapsigargin). Our previous analysis revealed that killing by these stimuli is complete only when levels of Ca^{2+} are adequate (29). This was for us a further suggestion that a Ca^{2+}-controlled pathway, like the calcineurin one, participates in killing by these stimuli. However, whether PPD1 simply slowed down the kinetics of death, or really prevented activation of the post-mitochondrial cascade was unclear. Notably, PPD1 allows clonogenic survival of cells treated with staurosporine, an indication that cells can still proliferate and that caspases are not activated in a vast majority of cells expressing PPD1. Such a powerful effect could be supported by the pleiotropic nature of a calcineurin inhibitor like PPD1. Conversely, our genetic analysis revealed that the mode of action of PPD1 is rather specific. Reed and colleagues showed that Ca^{2+}-mobilizing death stimuli induce activation of calcineurin. This in turn dephosphorylates the proapoptotic BH3-only molecule BAD, promotes its recruitment on mitochondria, triggering permeabilization of the outer membrane and cell death (32). On the other hand, PPD1 is equally antiapoptotic despite the lack of BAD. As expected, deletion of Bad had an inhibitory effect on killing by staurosporine, but expression of PPD1 in $Bad^{-/-}$ cells protected them as well as it did in their wild-type counterparts. Thus PPD1 does not inhibit death via an effect on calcineurin mediated dephosphorylation of BAD. In cells expressing a dominant negative Drp1, however, PPD1 has no further inhibitory effect, substantiating that it impinges on Drp1 to block apoptosis.

Our results further support the view that Drp1 and mitochondrial fragmentation participate in apoptosis. Indeed, previous works showed that Drp1 GTPase activity participated in cell death, since both a Drp1 mutant defective in GTPase activity due to the substitution of a lysine in the catalytic site for an alanine, K38ADrp1 (5), as well as a chemical inhibitor of the hydrolytic activity of the protein, mdivi-1 (34), were successful in delaying cell death. The precise mechanism by which inhibition of Drp1 affects progression of cell death remains to be elucidated. Apoptogenic factors such as Smac/DIABLO and AIF are still released even in the absence of Drp1, while cytochrome *c* is retained in the organelle (35, 36). As a matter of fact, cytochrome *c* is highly compartmentalized inside mitochondria, given its preferential localization inside the cristae (6, 37). This raises the possibility that the effects of blocking Drp1 in apoptosis are not a consequence of preservation of mitochondrial morphology, but of ultrastructure. This is confirmed by the finding that remodeling of the cristae, which mobilizes cytochrome *c*, is triggered by Drp1 and blocked by a dominant negative mutant of this dynamin (21). This scenario might be even more complicated since ablation of Drp1 causes compensatory changes in the cleavage of the inner membrane Opa1 (38), that controls cristae remodeling during apoptosis (9). In this respect, PPD1 and blockage of calcineurin, *i.e.* the Drp1 branch of apoptosis, could be an appealing mechanism to investigate, and in perspective to pharmacologically attack, since it does not interfere with the levels, but just with the mitochondrial translocation of the pro-fission dynamin-like protein.

Increasing evidence indicates that mitochondrial fragmentation is a hallmark of several diseases associated with increased apoptosis, from Alzheimer's (39) to Parkinson's (40) disease. Thus, the checkpoint controlled by calcineurin could serve as a potential therapeutic target by compounds or small peptides that mimic the action of PPD1.

Materials and Methods

Subcellular Fractionation, Immunoprecipitation, and Immunoblotting.

Subcellular fractionation was performed as described in (41). For immunoprecipitation experiments, cells were lysed in CPBS buffer (6mM CHAPS in PBS,pH7.4). Lysates were incubated with the anti-Drp1 antibody (1:50, 14 h, 4 °C) and the protein-antibody complex was precipitated by centrifugation after incubation with protein G-coated magnetic beads (Dynal, 2 h, 4 °C). The immunoprecipitated material was washed twice in CPBS and resuspended in SDS/PAGE loading buffer (NuPAGE), boiled, and loaded on 4–12% gels (NuPAGE). For immunoblotting, proteins were transferred onto polyvinylidene fluoride (Millipore) membranes and probed with the following antibodies: α-Drp1 (BD, 1:2000); α-cyclophilinA (Upstate, 1:2000);

Isotype-matched, horseradish peroxidase-conjugated secondary antibodies (Amersham) were used, followed by detection by chemiluminescence (Amersham).

Calcineurin Activity Assay.

Cells grown on 24-mm wells incubated in HBSS buffer were treated as indicated, washed several times with TBS buffer (10mM Tris-Cl, pH 7.4, 150mMNaCl), resuspended in TBS, and lysed in CTBS buffer (6mM CHAPS in TBS, pH 7.4). Calcineurin activity was determined using an *in vitro* assay kit and following manufacturer instructions (Calbiochem). For comparative reasons, basal calcineurin activity in untreated cells was set to 100%.

Imaging.

Imaging of mitochondrial membrane potential in HeLa cells was performed as previously described (31). Data are graphed as percentage of the initial value for comparative reasons. For confocal imaging, 10^5 cells seeded onto 24 mm-round glass coverslips were

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transfected as indicated and after 24 h were incubated in HBSS supplemented with 10 mM Hepes and placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer Ultraview LCI confocal system, a piezoelectric z-axis motorized stage (Pifoc, Physik Instrumente), and a Orca ER 12-bit CCD camera (Hamamatsu Photonics). Cells expressing mtRFP were excited using the 568-nm line of the He-Ne laser (Perkin–Elmer) with exposure times of 100 msec using a 60x1.4 NA Plan Apo objective (Nikon). In time-course experiments, images were acquired every 10 sec for 40 min. Quantitative analysis of mitochondrial shape changes was performed by evaluating the time at which cells displayed fragmented mitochondria after addition of the inducer. Organelles were classified as fragmented when 50% of the total cellular mitochondria displayed a major axis <5 μm.

For cytochrome *c* immunolocalization, cells grown on coverslips were transfected with mtRFP and after 24 hr incubated as detailed. Immunostaining for cytochrome *c* was performed as described in (29). For cytochrome *c* and mtRFP detection green and red channel images were acquired simultaneously using two separate color channels on the detector assembly of a Nikon Eclipse E600 microscope equipped with a Biorad MRC-1024 laser scanning confocal imaging system. The localization index was calculated as described in (31)

Analysis of Cell Death

1×10^5 HeLa cells or MEFs grown in 12-well plates were cotransfected with pEGFP and the indicated vectors. At the time points indicated, cells were treated as described and stained with Annexin-V-Alexa568 (Roche) according to manufacturer's protocol. Apoptosis was measured by flow cytometry (FACSCalibur) as the percentage of annexin-V-positive events in the GFP-positive population.

Clonogenic assay

1×10^6 HeLa cells seeded on 10 cm Petri dishes were cotransfected with pEGFP and PPD1 or empty vector. After 24 hrs cells were sorted by FACS and 50000 GFP-positive were treated with 2 µM staurosporine in DMSO or DMSO alone. After 5 hrs cells were washed with PBS, trypsinized, resuspended in complete DMEM (DMEM, Invitrogen, supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% non essential amino acid mix (Gibco), 1 mM penicillin/streptomycin) and plated on 10 cm Petri dishes containing 15 ml of DMEM supplemented with 2mM glutamine, 1mM penicillin/streptomycin, 20% fetal bovine serum and 0.3% Bacto Agar (Invitrogen). Plates were incubated at 37°C in a fully humidified atmosphere of 95% air and 5% CO_2. After 12 days, colonies were visualized by 0.1% crystal violet in 20% ethanol.

Acknowledgements

Reference List

1. Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15

2. Danial NN and Korsmeyer SJ (2004) Cell death: critical control points. Cell 116:205-219

3. Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B and Andrews DW (2008) Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell 135:1074-1084

4. Martinou I, Desagher S, Eskes R, Antonsson B, Andre E, Fakan S and Martinou JC (1999) The release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. J. Cell Biol. 144:883-889

5. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL and Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell 1:515-525

6. Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA and Korsmeyer SJ (2002) A Distinct Pathway Remodels Mitochondrial Cristae and Mobilizes Cytochrome c during Apoptosis. Dev. Cell 2:55-67

7. Wasilewski M and Scorrano L (2009) The changing shape of mitochondrial apoptosis. Trends Endocrinol. Metab 20:287-294

8. Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L and De Strooper B (2006) Mitochondrial Rhomboid

PARL Regulates Cytochrome c Release during Apoptosis via OPA1-Dependent Cristae Remodeling. Cell 126:163-175

9. Frezza C, Cipolat S, Martins dB, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B and Scorrano L (2006) OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion. Cell 126:177-189

10. de Brito OM and Scorrano L (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 456:605-610

11. Smirnova E, Griparic L, Shurland DL and van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell 12:2245-2256

12. Yoon Y, Krueger EW, Oswald BJ and McNiven MA (2003) The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol. Cell Biol. 23:5409-5420

13. Cereghetti GM and Scorrano L (2006) The many shapes of mitochondrial death. Oncogene 25:4717-4724

14. Cereghetti GM, Stangherlin A, Martins dB, Chang CR, Blackstone C, Bernardi P and Scorrano L (2008) Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. U. S. A 105:15803-15808

15. Karbowski M, Lee YJ, Gaume B, Jeong SY, Frank S, Nechushtan A, Santel A, Fuller M, Smith CL and Youle RJ (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. 159:931-938

16. Arnoult D, Rismanchi N, Grodet A, Roberts RG, Seeburg DP, Estaquier J, Sheng M and Blackstone C (2005) Bax/Bak-dependent release of DDP/TIMM8a promotes Drp1-mediated mitochondrial fission and mitoptosis during programmed cell death. Curr. Biol. 15:2112-2118

17. Frezza C, Cipolat S, Martins dB, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B and Scorrano L (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 126:177-189

18. Yuan H, Gerencser AA, Liot G, Lipton SA, Ellisman M, Perkins GA and Bossy-Wetzel E (2007) Mitochondrial fission is an upstream and required event for bax foci formation in response to nitric oxide in cortical neurons. Cell Death. Differ. 14:462-471

19. Parone PA, James DI, Da Cruz S, Mattenberger Y, Donze O, Barja F and Martinou JC (2006) Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis. Mol. Cell Biol. 26:7397-7408

20. Arnoult D, Rismanchi N, Grodet A, Roberts RG, Seeburg DP, Estaquier J, Sheng M and Blackstone C (2005) Bax/Bak-dependent release of DDP/TIMM8a promotes Drp1-mediated mitochondrial fission and mitoptosis during programmed cell death. Curr. Biol. 15:2112-2118

21. Germain M, Mathai JP, McBride HM and Shore GC (2005) Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. EMBO J. 24:1546-1556

22. Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR and Nunnari J (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev. Cell 14:193-204

23. Sheridan C, Delivani P, Cullen SP and Martin SJ (2008) Bax- or Bak-induced mitochondrial fission can be uncoupled from cytochrome C release. Mol. Cell 31:570-585

24. Cereghetti GM, Stangherlin A, Martins dB, Chang CR, Blackstone C, Bernardi P and Scorrano L (2008) Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. U. S. A 105:15803-15808

25. Galigniana MD, Morishima Y, Gallay PA and Pratt WB (2004) Cyclophilin-A is bound through its peptidylprolyl isomerase domain to the cytoplasmic dynein motor protein complex. J. Biol. Chem. 279:55754-55759

26. Varadi A, Johnson-Cadwell LI, Cirulli V, Yoon Y, Allan VJ and Rutter GA (2004) Cytoplasmic dynein regulates the subcellular distribution of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1. J. Cell Sci. 117:4389-4400

27. Galigniana MD, Radanyi C, Renoir JM, Housley PR and Pratt WB (2001) Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. J. Biol. Chem. 276:14884-14889

28. Cereghetti GM, Stangherlin A, Martins de BO, Chang CR, Blackstone C, Bernardi P and Scorrano L (2008) Dephosphorylation by calcineurin regulates

translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. U. S. A 105:15803-15808

29. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T and Korsmeyer SJ (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300:135-139

30. Lee YJ, Jeong SY, Karbowski M, Smith CL and Youle RJ (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol. Biol. Cell 15:5001-5011

31. Petronilli V, Penzo D, Scorrano L, Bernardi P and Di Lisa F (2001) The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings in situ. J. Biol. Chem. 276:12030-12034

32. Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF and Reed JC (1999) Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284:339-343

33. Puthalakath H, Huang DC, O'Reilly LA, King SM and Strasser A (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell 3:287-296

34. Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR and Nunnari J (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev. Cell 14:193-204

35. Estaquier J and Arnoult D (2007) Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. Cell Death. Differ. 14:1086-1094

36. Parone PA, James DI, Da Cruz S, Mattenberger Y, Donze O, Barja F and Martinou JC (2006) Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis. Mol. Cell Biol. 26:7397-7408

37. Bernardi P and Azzone GF (1981) Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. J. Biol. Chem. 256:7187-7192

38. Mopert K, Hajek P, Frank S, Chen C, Kaufmann J and Santel A (2009) Loss of Drp1 function alters OPA1 processing and changes mitochondrial membrane organization. Exp. Cell Res. 315:2165-2180

39. Wang X, Su B, Lee HG, Li X, Perry G, Smith MA and Zhu X (2009) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J. Neurosci. 29:9090-9103

40. Lutz AK, Exner N, Fett ME, Schlehe JS, Kloos K, Lammermann K, Brunner B, Kurz-Drexler A, Vogel F, Reichert AS, Bouman L, Vogt-Weisenhorn D, Wurst W, Tatzelt J, Haass C and Winklhofer KF (2009) Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. J. Biol. Chem. 284:22938-22951

41. Frezza C, Cipolat S and Scorrano L (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat. Protoc. 2:287-295

Legend to figures

Figure 1. **PPD1 delays mitochondrial fragmentation induced by mitochondrial depolarization.** (a) Changes in mitochondrial fluorescence of TMRM. HeLa cells loaded with TMRM were treated where indicated (arrows) with 20 µM ArA or 2 µM FCCP. Fluorescence intensities were normalized to the initial value for comparative reasons. Data represent mean ± SE of 4 independent experiments. (b) Quantitative analysis of mitochondrial shape changes observed by real time confocal imaging of HeLa cells previously transfected with PPD1 or empty vector. At t=3 min, cells were treated as indicated with 20 µM ArA or 2 µM FCCP. Morphometry was performed as described (40). Data represent mean ± SE of 8 different experiments. In each experiment, 20 cells were scored. (c) Representative frames acquired at indicated times from real time confocal imaging of HeLa cells treated exactly as described in (b). Bar, 20 µm.

Figure 2. **PPD1 displaces Drp1 from cyclophilin A and inhibits calcineurin activation induced by mitochondrial dysfunction.** (a) HeLa cells transfected as indicated were lysed and 200 µg of protein were immunoprecipitated using the indicated antibody (concentration). 100 µg were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Where indicated, cells were treated with ArA (20 µM, 20 min at RT) (b) Calcineurin activity was measured in cytosolic extracts of HeLa cells transfected with PPD1 or empty vector and treated where indicated for 15 min with 20 µM ArA or 2 µM FCCP. Data are compared to the activity of untreated cells set as 100% and represent mean ± SE of 4 independent experiments.

Figure 3. **PPD1 prevents cell death.** HeLa cells cotransfected with pEGFP and PPD1 or empty vector were treated for the indicated time with staurosporine (2 µM), etoposide (2

µM), hydrogen peroxide (1 mM) or thapsigargin (2 µM), collected and stained with Annexin V. Viability was analyzed by flow cytometry as the percentage of annexin-V-positive events in the GFP-positive population. Data represent mean ± SE of 5 independent experiments.

Figure 4. PPD1 acts at mitochondria to block apoptosis. (a) Representative frames acquired at indicated times from real time confocal imaging of HeLa cells treated with staurosporine (2 µM) 24 h after cotransfection with mtRFP and the indicated plasmid. Bar, 20 µm. (b) Representative images of subcellular cytochrome c distribution. HeLa cells were cotransfected with mtRFP (red) and the indicated plasmid. Where indicated, cells were treated for 2 h with staurosporine (2 µM), fixed and immunostained for cytochrome c (green). Bar, 20 µm. (c) Localization index of cytochrome c. Experiments were performed as in (b). Data represent mean ± SE of 3 independent experiments (d) Clonogenic survival assay. HeLa cells cotransfected with peGFP and the indicated plasmid were treated as indicated (staurosporine 2 µM, 5 h), sorted for GFP-positivity by flow cytometry and plated. After 12 days colonies were visualized by staining with 0.1% crystal violet in 20% ethanol. (e) Quantitative analysis of the effect of PPD1 on clonogenic survival of STS treated cells. Experiments were exactly as in (d) Data represent mean ± SE of 3 different experiments.

Figure 5. PPD1 blocks apoptosis by inhibiting Drp1. (a) MEFs cotransfected with pEGFP and the indicated plasmids were treated with staurosporine (2 µM) for the indicated times and viability was determined by flow cytometry in the GFP posiive population. Data represent mean ± SE of 6 independent experiments. (b) MEFs of the indicated genotype were cotransfected with pEGFP and the indicated plasmids and treated with staurosporine (2 µM) for the indicated time. Viability was determined as in (a). Data are mean ± SE of 6 independent experiments.
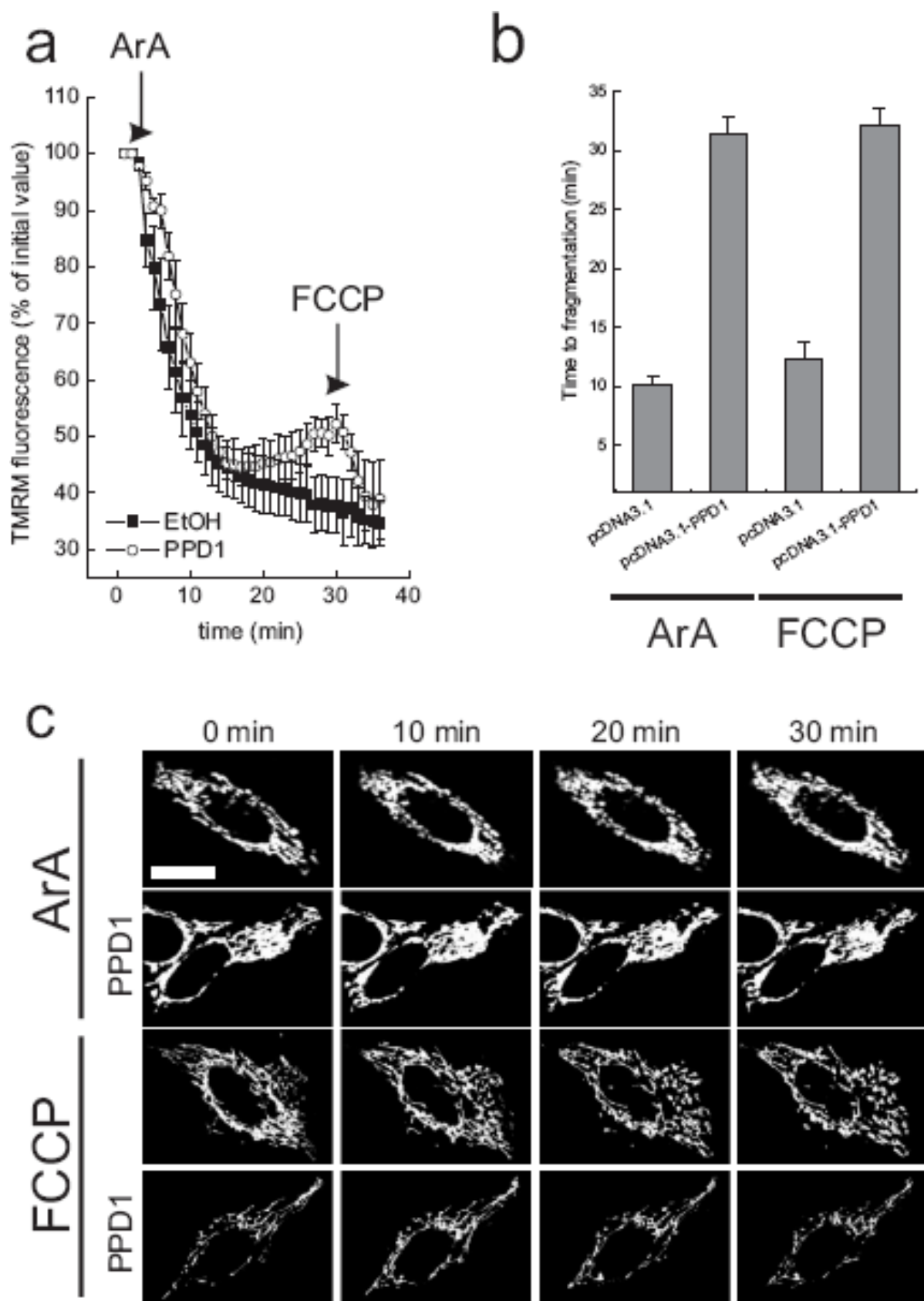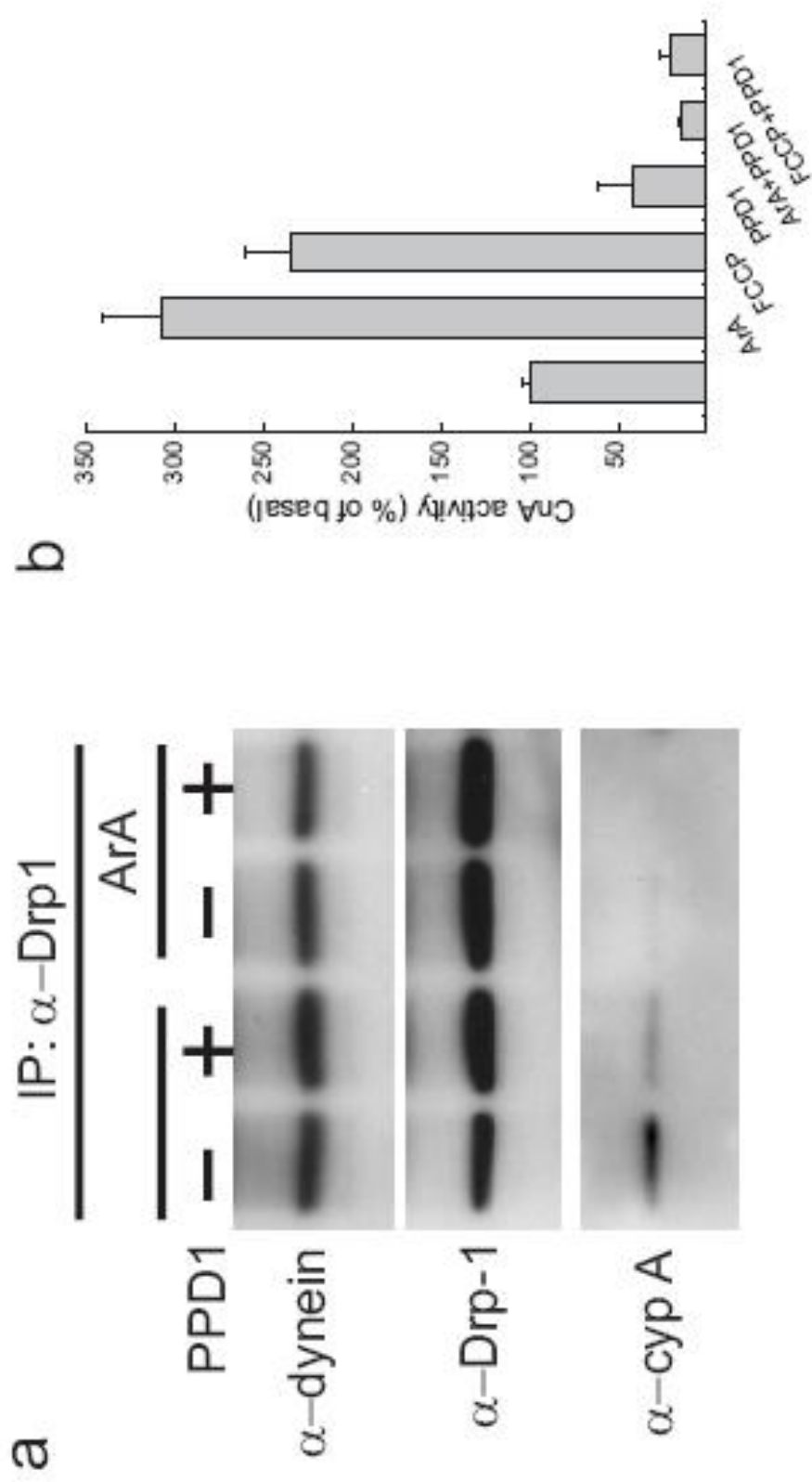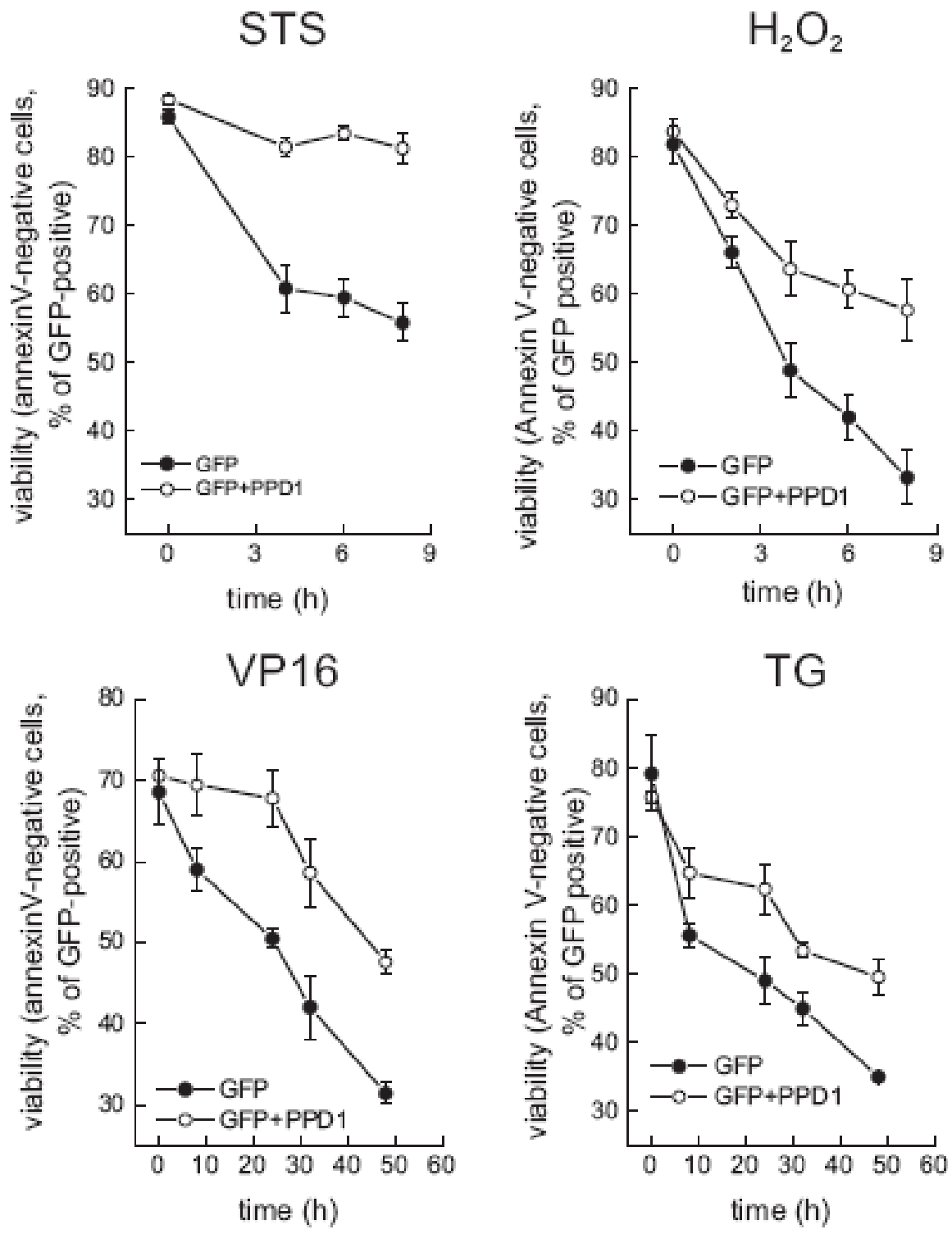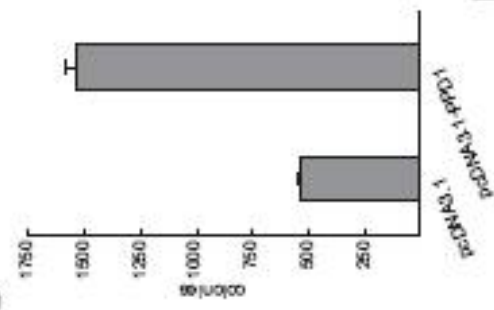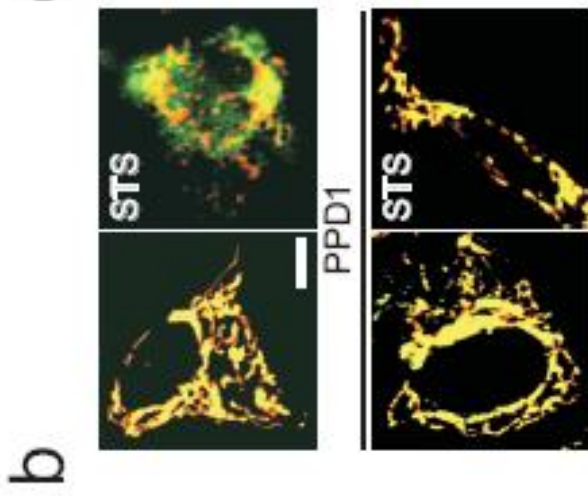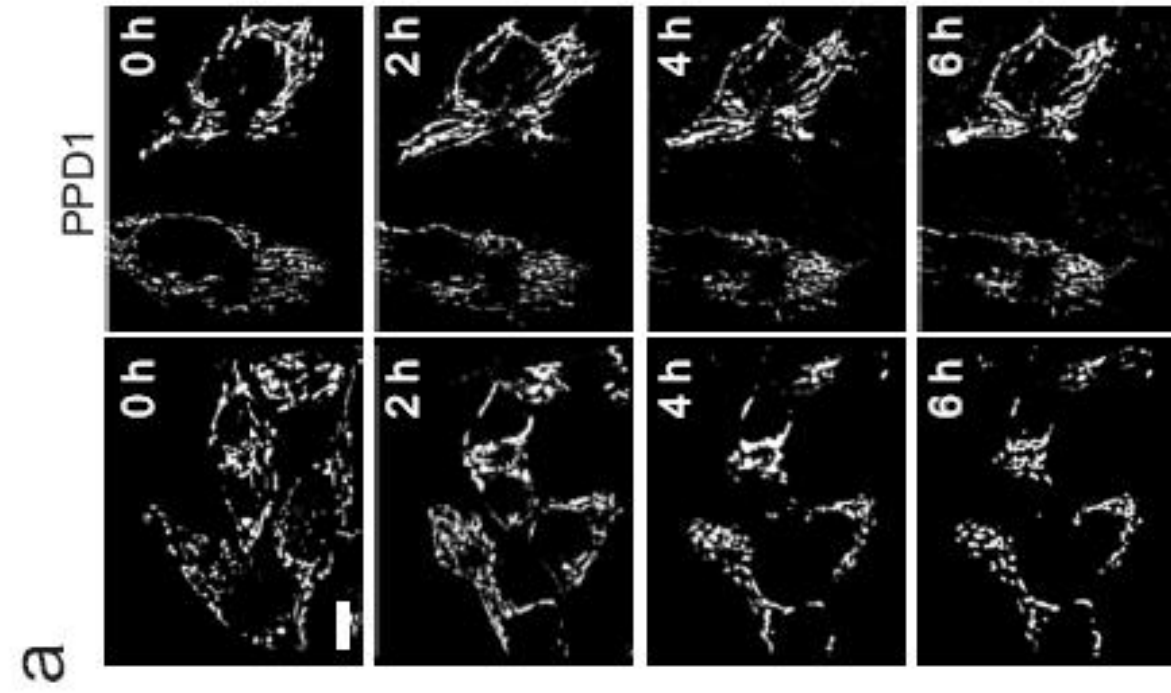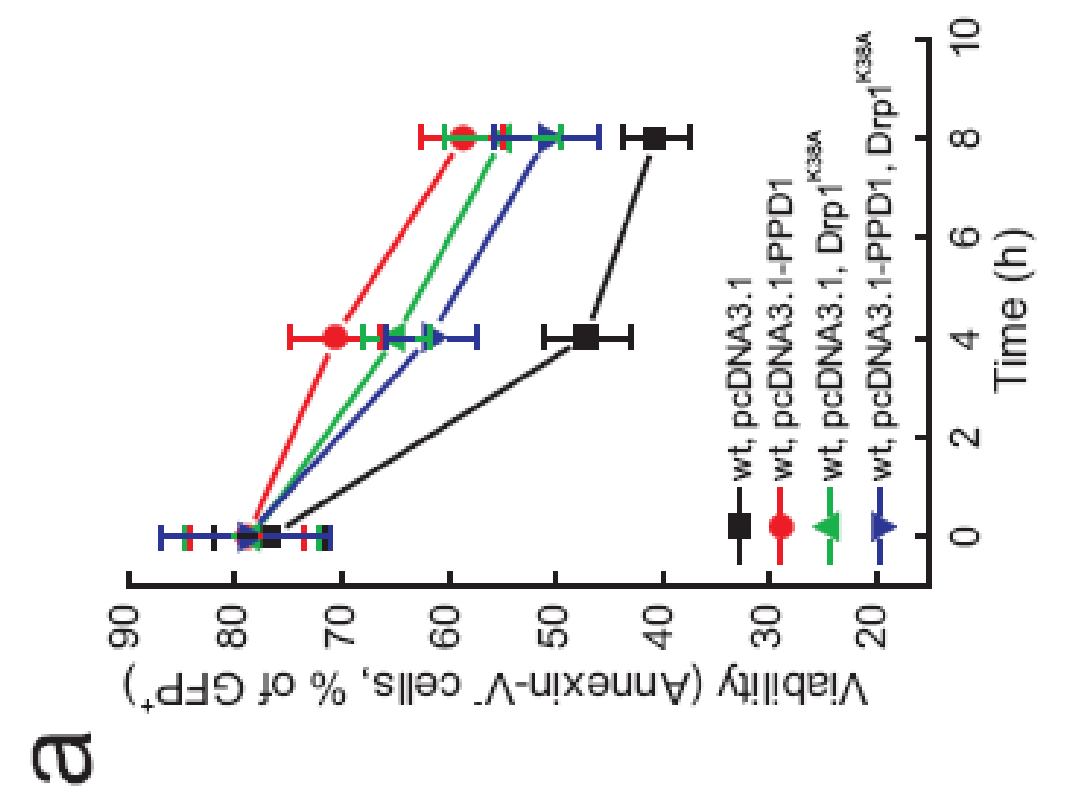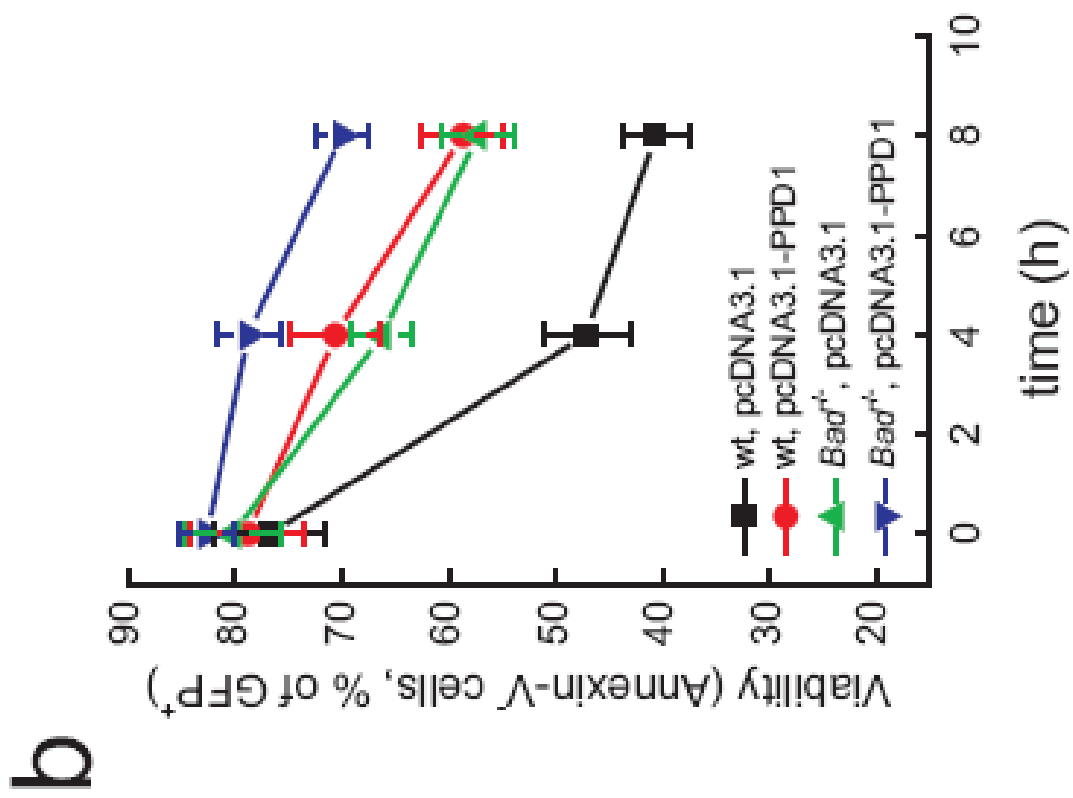
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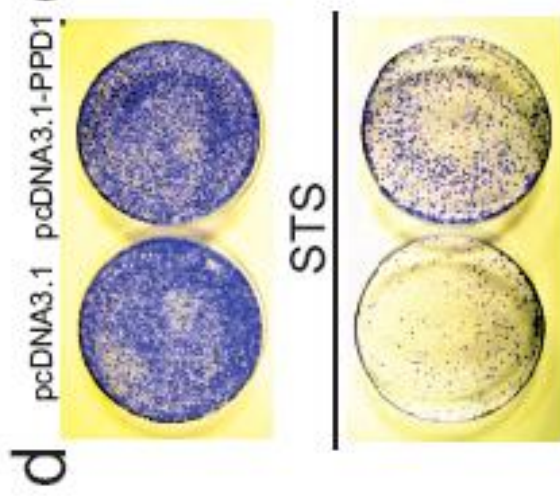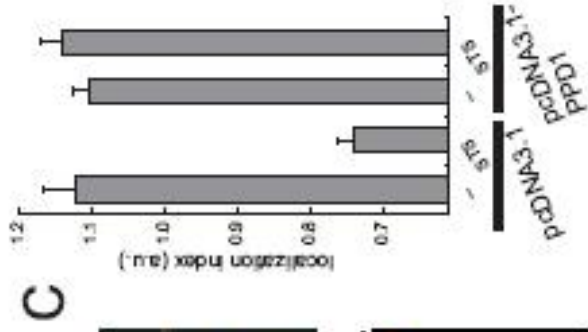
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Mitochondrial fission and cristae disruption sustain apoptosis in cellular models of Huntington's disease.

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Abstract

Huntington's Disease, a genetic neurodegenerative disease caused by a polyglutamine expansion in the Huntingtin protein is accompanied by multiple mitochondrial alterations. Here we show that in cells from patients and from a knock-in mouse model of Huntington's disease the mitochondrial fission machinery is hyperactive, causing alterations in the cristae structure and therefore leading to increased cytochrome c release and apoptosis. In Huntington's disease cells, mitochondrial translocation and activation of the pro-fission protein Drp1 is increased, leading to mitochondrial fragmentation. In these fragmented mitochondria, the oligomers of Opa1 are more easily disrupted upon apoptotic stimulation. Accordingly, cytochrome c release and apoptosis are faster. Enforced expression of Opa1 or prevention of Drp1 activation ameliorate mitochondrial morphology and ultrastructure, and ultimately cell death. In conclusion, we elucidate a feed forward mechanism of mitochondrial fission and cristae remodelling predisposing mitochondria to apoptotic damage in Huntington's disease.

Introduction

Mitochondria are key organelles for the life and death of the cell. They serve as platforms that sense damage and amplify it by releasing cytochrome c and other cofactors in the cytoplasm in order to activate effector caspases that accomplish the demise of the cell (Danial & Korsmeyer, 2004). This release is tightly controlled by proteins of the Bcl-2 family, is sustained by the permeabilization of the outer mitochondrial membrane and is accompanied by changes in the morphology and ultrastructure of mitochondria (Wasilewski & Scorrano, 2009). Remodelling of the mitochondrial cristae, with widening of their narrow tubular junctions (Scorrano et al, 2002), and fragmentation of the mitochondrial network (Frank et al, 2001), both required for the complete release of cytochrome c and the progression of apoptosis, can cross-talk (Germain et al, 2005). Mitochondrial morphology in living and dying cells is controlled by a growing family of mitochondria-shaping proteins. The core components of this machinery include both pro-fission (the cytoplasmic dynamin related protein 1, Drp1; and its mitochondrial receptor fission-1, Fis1) and pro-fusion (the large GTPases Optic Atrophy 1, Opa1, in the inner membrane and Mitofusin, Mfn, 1 and 2 in the outer mitochondrial membrane) proteins (Liesa et al, 2009).

In many degenerative diseases, mitochondria are more susceptible to apoptotic stimuli (Thompson, 1995). This is particularly evident in neuronal tissues, characterized by high energy demands to maintain proper functions (Chan, 2006) and for example unable to switch to glycolysis when mitochondrial oxidative phosphorylation is impaired. In keeping with the importance of mitochondrial shape regulation for the progression of cell death, several neurodegenerative diseases are associated with mutations in the genes coding for mitochondria shaping proteins. Mutations in Opa1 cause dominant optic atrophy (Alexander et al, 2000; Delettre et al, 2000), while mutations in Mfn2 are linked to Charcot-Marie-Tooth IIa (Zuchner et al, 2004). Mutations in other accessory mitochondria-shaping proteins like GDAP1 are associated with other neurodegenerative diseases (Niemann et al,

2005) and considerable interest was recently captured by the potential role of mitochondrial morphology changes as the pathogenic mechanism for familial forms of Parkinson's disease (PD) caused by mutations in the *PINK1* and *PARKIN* genes (Poole et al, 2008), albeit it is unclear whether the defect is primary (Lutz et al, 2009) or a consequence of mitochondrial dysfunction (Gautier et al, 2008; Morais et al, 2009). Altogether, there is a general consensus that mitochondrial dynamics is a key process for neurons, where it controls not only survival (Chen et al, 2007), but also synaptogenesis and formation of dendritic spines (Li et al, 2004). Thus, mitochondrial alterations are intensely studied as potential key components in the natural history of neurodegenerative conditions, including Huntington's disease (HD).

HD is an autosomal dominant neurodegenerative disease caused by the expansion beyond 36 of a CAG repeat in the IT15 gene (4p16.3) (The Huntington's Disease Collaborative Research Group, 1993). HD is characterized clinically by variables age of onset (normally between 40 and 50) and severity that correlate directly with the length and the gene dosage of the CAG repeat number (Duyao et al, 1993) . HD patients are affected by neurological (choreoathetosis, psychiatric disturbances, cognitive defects) and extraneurological (wasting, immunological and cardiological defect) alterations and ultimately die 10-20 years from the onset of the disease (Martin & Gusella, 1986) . The key pathological feature of HD is the progressive loss of neurons with atrophy and gliosis of the basal ganglia and the cortex, especially of the GABAergic spiny neurons of the striatum (Ferrante et al, 1991). The IT15 gene encodes for the ubiquitous protein Huntingtin (Htt), and the CAG repeats result in the expansion of an N-terminal polyglutamine trait (Schilling et al, 1995; Sharp et al, 1995). Htt is a large protein of 350 kDa with no homology with other known proteins, located in the cytoplasm and found associated with a variety of subcellular structures, from Golgi to the endoplasmic reticulum, to mitochondria, to the nucleus where it exerts transcriptional effects (Difiglia et al, 1995; Gutekunst et al, 1995; De Rooij et al, 1996; Kegel et al, 2002; Panov et al, 2002). Htt is required during development (Zeitlin et al, 1995)

and is subjected to post-translational modifications, including phosphorylation and cleavage, that are important for the pathogenesis of HD (Wellington et al, 2000; Hackam et al, 1998; Graham et al, 2006; Pardo et al, 2006; Gu et al, 2009).

The exact pathobiology of HD remains elusive. Several theories have been put forward to explain how mutated Htt is neurotoxic: they range from altered transcriptional activity (Sugars & Rubinsztein, 2003) to impaired intracellular trafficking (Gunawardena et al, 2003; Trushina et al, 2004) to the formation of aggregates (Difiglia et al, 1997) that clog the proteasomal (Jana et al, 2001) and autophagic machineries (Ravikumar et al, 2004), to the hypersensitivity to excitotoxicity (Fernandes et al, 2007). Irrespective of the apical mechanism, the key feature of HD remains the death of the GABAergic neurons of the striatum, calling for a crucial role of mitochondria in the process. This is substantiated by a number of experimental evidence pointing to altered mitochondrial Ca^{2+} buffering capacity (Panov et al, 2002), altered mitochondrial bioenergetics (Grunewald & Beal, 1999), increased susceptibility of cells derived from animal models of HD to excitotoxicity by N-methyl-D-Aspartate (NMDA) receptor activation (Zeron et al, 2002). These changes are not only observed in cells from HD patients or from animal models of HD, but also recapitulated in vitro or by the expression of mutant Htt (Panov et al, 2002; Choo et al, 2004). The N-terminus of mutant Htt , whose role in inducing striatal dysfunction is well established (Gu et al, 2009), can indeed colocalize with mitochondria and is probably responsible for the retrieval of full length Htt on the organelle (Orr et al, 2008). In addition, administration of the toxin 3-nitropropionic acid (3NPA), a well known inhibitor of complex II, recapitulates the features of HD and is widely used to model the disease in the animal and in vitro (Beal et al, 1993; Almeida et al, 2004). Interestingly, 3NPA has been recently reported to cause mitochondrial morphological abnormalities in primary cortical neurons (Liot et al, 2009) and expression of the N-terminal portion of Htt bearing polyglutamine repeats in HeLa cells causes mitochondrial fragmentation, suggesting that altered mitochondrial dynamics may participate in the pathobiology of HD (Wang et al, 2009). Finally,

lymphoblasts from HD patients display striking mitochondrial ultrastructural abnormalities that almost completely phenocopy the images of cristae remodelling observed in apoptotic mitochondria (Mormone et al, 2006).

Here we report that different cellular models of HD constitutively expressing mutant Htt at physiological levels (lymphoblasts from patients or striatal cells from knock-in mice) display mitochondrial fragmentation and disruption of the cristae. These changes result from hyperactivity of Drp1, lead to increased susceptibility to cytochrome c release and cell death and can be reverted by preventing Drp1 activation or stabilizing mitochondrial cristae.

Results

Mitochondrial fragmentation and cristae disruption in cellular models of HD is associated with increased mitochondrial levels of Drp1.

Lymphoblasts from HD patients bearing a heterozygous 48 polyglutamine repeat (48Q) display striking abnormalities of mitochondrial ultrastructure (Mormone et al, 2006). In order to verify if this was accompanied also by changes in mitochondrial morphology we expressed in these cells and in lymphoblasts from age and gender matched healthy controls a mitochondrially targeted yellow fluorescent protein (mtYFP). Three-D surface rendered images of confocal z-stacks corresponding to the whole cellular volume revealed that in 48Q lymphoblasts mitochondria were fragmented and clustered, as opposed to the network of interconnected organelles retrieved in the healthy donor. When we extended our analysis to lymphoblasts from a patient with a longer repeat (70Q) or from another patient carrying a homozygous mutation with 45 and 47 repeats (45+47Q) we observed a length and gene dosage dependent increase in mitochondrial fragmentation (Fig. 1A,B). To verify if this was observed also in neuronal cell, we turned to two clonal striatal progenitor cell lines isolated from knock-in Hdh^{Q111} mouse embryos bearing a 111 polyglutamine repeat (Q111/0 and Q111/1) (Trettel et al, 2000). Mitochondrial fragmentation and clustering also characterized these cells, Q111/0 showing a more severe phenotype (Fig. 1C,D). The morphological defect was retained also when we induced neuronal differentiation (Fig. S1). We then turned to an ultrastructural analysis of the striatal cell lines and found that the increased fragmentation was accompanied by a loss of the cristae that are conversely well developed in the cell line isolated from the wt animal (Fig. 1E). Thus, these experiments show that mitochondrial fragmentation and disruption of cristae is a hallmark of cells from HD patients and a mouse model of the disease.

To understand the molecular basis of the observed fragmentation, we measured levels of mitochondria-shaping proteins in the different HD cellular models, without noticing any relevant change in pro-

fusion or pro-fission proteins (Fig. 2A,B). We then turned to isolated mitochondria and monitored levels of pro-fission Drp1, that once dephosphorylated translocates to mitochondria (Cereghetti et al, 2008). Interestingly, we consistently found more Drp1 associated with HD mitochondria, irrespective of the cellular model they were isolated from. Furthermore, increased levels of Drp1 were retrieved on mitochondrial from the Q111/0 clone and from the lymphoblasts from the 45+47Q patient, which displayed the most severe fragmentation phenotype (Fig. 2 C,D). The increased levels of mitochondrial Drp1 were also more active, as judged by the abundance of Drp1 homo-oligomers following cross-linking (Fig. 2E-G) (Zhu et al, 2004). Thus, HD-associated fragmentation can result from increased mitochondrial levels and activity of the pro-fission Drp1.

Genetic and pharmacological correction of mitochondrial fragmentation in HD models

Mitochondrial morphology is the outcome of the balance between fusion and fission processes, and increased fission for example can be counterbalanced by enhancing mitochondrial fusion. We therefore verified if we could restore mitochondrial morphology to normal levels by expressing pro-fusion proteins in lymphoblasts from HD patients. Ectopic expression of the pro-fusion protein Opa1 (Cipolat et al, 2004) together with the mitochondrial marker mtYFP fully corrected mitochondrial fragmentation in 48Q, 70 Q (Fig. 3A,B) and in 45+47Q (Fig. 3C,D) lymphoblasts, as judged by our 3D confocal imaging approach. The correction was also observed in the striatal cell lines Q111/1 and Q111/0 (Fig. 3E,F). As expected, Opa1 also caused mitochondrial elongation in the lymphoblasts from the corresponding healthy donors and in the striatal cell line from the wt mouse (Fig. 3). We then decided to further explore which other manoeuvres could be devised to correct the severe mitochondrial fragmentation of the 45+47Q lymphoblast and of the striatal HD cell lines. As expected, mitochondrial elongation was restored when we expressed the pro-fusion partner of Opa1, Mfn1 (whose levels are below the immunoblot detection limit in all the cell line tested, not shown), or a dominant negative

mutant of Drp1 (Fig. 3C-F). We then reasoned that if mitochondrial fragmentation was sustained by increased mitochondrial levels of Drp1, we could also attempt to restore morphology of the organelle by interfering with its mitochondrial localization by expressing a dominant negative calcineurin mutant that blocks its mitochondrial translocation (Cereghetti et al, 2008). Notably, this mutant was able to fully compensate mitochondrial fragmentation in both the lymphoblast and the striatal cell models of HD (Fig. 3C-F). The importance of calcineurin in the process was further reinforced by the ability of its pharmacological inhibitor FK506 to ameliorate mitochondrial morphology in the HD striatal models (Fig. 3E,F). In sum, these data demonstrate that mitochondrial morphology can be corrected in the HD cell lines by enforcing fusion or by blocking fission. Notably, blockage of calcineurin is one of the approaches that restored mitochondrial elongation, suggesting a role for this phosphatase in organelle fragmentation in the HD models.

Increased apoptosis in HD cell models: the role of Opa1-dependent cristae remodelling

We next ought to determine if the observed mitochondrial fragmentation was associated with increased susceptibility to cell death by intrinsic stimuli that engage the mitochondrial pathway. To this end we measured exposure of phosphatidylserine in lymphoblasts or activation of effector caspases in striatal precursors by monitoring cleavage of the caspases substrate PARP. Irrespective of the method used, all the HD cell models were more sensitive to death by staurosporine (Fig. 4) as well as by other stimuli tested (hydrogen peroxide, etoposide, not shown). Interestingly, levels of death seemed to correlate with the extent of mitochondrial fragmentation, even if some unavoidable variability was observed especially in the human healthy donor control cell lines (Fig. 4A). Why are HD cells, displaying fragmented mitochondria, more sensitive to apoptosis? We excluded that this was a consequence of lower levels of anti- (Bcl-2, Bcl-XL), or higher levels of proapoptotic (Bax, Bak) Bcl-2 family members (not shown). We then verified if the increased death was associated with changes in the

mitochondrial pathway of apoptosis. To this end, we compared the rate of cytochrome c release from mitochondria of the striatal precursors *in situ* by applying the semiquantitative localization index we previously employed (Frezza et al, 2006). Irrespective of the stimulus used, mitochondria from the cell bearing the 111Q repeat released cytochrome c faster (Fig. 5A,B). This was not caused by a faster activation of Bax or Bak (not shown). We then wanted to verify if this increased susceptibility was somehow "memorized" by mitochondria outside of their HD cell context. We therefore compared cytochrome c release in response to recombinant BID from mitochondria purified from the lymphoblasts of the HD patients and of their matched healthy donors. Interestingly, mitochondria isolated from the HD lymphoblasts were more susceptible to BID than their control counterparts (Fig. 5C,E,G). Also in this case, oligomerization of Bax and Bak was comparable, ruling out that the faster release was the consequence of increased activation of the multidomain proapoptotics required for the release of cytochrome c from mitochondria (Wei et al, 2001). We then reasoned that the increased release could reflect alterations in the Opa1-controlled cristae remodelling pathway. We verified if in HD mitochondria the disruption of the Opa1 containing oligomers that keep cristae junctions in check (Frezza et al, 2006) was affected. Interestingly, disruption of the oligomers correlated perfectly with the rate of cytochrome c release in mitochondria isolated from the HD lymphoblasts (Fig. 5D,F,H), supporting a role for changes in Opa1 oligomerization in the response of HD mitochondria to apoptotic stimuli. We similarly found that Opa1 oligomers were less represented and more easily disrupted in situ in the HD striatal precursors than in their wt counterpart (Fig. S2A). Interestingly, in the HD striatal cells we also noted increased cleavage of Opa1 to short forms following apoptotic stimulation, leading to an unbalance between the long and the short forms both required for the formation of the Opa1 oligomer (Frezza et al, 2006) and for competent mitochondrial fusion (DeVay et al, 2009). We next wished to verify if the changes in Opa1 oligomerization were accompanied by ultrastructural alterations of mitochondria. We therefore performed a morphometric analysis by electron microscopy

of mitochondrial cristae in wt, Q111/1 and Q111/0 striatal precursors early (3 hrs) following induction of apoptosis. The length of the cristae were reduced upon treatment with staurosporine, more in the HD cells than in their wt counterpart (Fig. 6A and quantification in B). This further substantiated a role for ultrastructural alterations of HD mitochondria in their increased susceptibility to apoptosis.

Correction of HD mitochondrial phenotype by Opa1 or by blockage of fission.

Our results pointed to a role for cristae morphology in the increased apoptotic susceptibility of HD cellular models. We therefore decided to address if stabilization of Opa1 oligomers, by enforced expression of Opa1, could correct the defects of the HD cells. Expression of Opa1 rescued cell death in the lymphoblasts from HD patients (Fig. 7A). Similarly, when we turned to the Q111/0 clone which displays the most severe defects in terms of mitochondrial morphology, apoptosis and ultrastructural changes, we could completely complement it by enforced expression of Opa1 (Fig. 7B). We then verified if also the pro-fusion Mfn1, that like Opa1 corrects mitochondrial fragmentation in HD cells (Fig. 3), was able to revert the apoptotic phenotype. Of note, the lymphoblasts from the homozygous HD patient, or the Q111/0 striatal clone were not protected from apoptosis upon expression of Mfn1 (Fig. 7 A, D). Thus, enforced fusion is not sufficient to correct the mitochondrial apoptotic phenotype of HD cellular models.

At this point we were left with the conundrum of how mitochondrial fragmentation observed in the HD cell models and sustained by increased mitochondrial levels and activity of Drp1 (Fig. 2) resulted in increased susceptibility to apoptosis. We hypothesized that this could be a consequence of the cristae changes that can be triggered by the activation of Drp1 (Germain et al, 2005). We tested this possibility by addressing if blockage of Drp1 was able to revert the apoptotic phenotype and the changes in the cristae shape. Expression of a dominant negative mutant of Drp1 was able to protect from death the lymphoblasts from the homozygous HD patient, or the Q111/0 striatal clone (Fig. 7A,C). Furthermore,

the calcineurin inhibitor FK506 that prevents translocation of Drp1 to mitochondria (Cereghetti et al, 2008) was similarly effective in preventing activation of caspases in the striatal clone (Fig. 7E). When we then turned to the morphometric analysis of the mitochondrial cristae in the striatal clones, we found that expression of Opa1 was expectedly able to correct the disruption of the mitochondrial ultrastructure. This was phenocopied by the dominant negative mutant of Drp1 (Fig. 6A,B), further substantiating a model in which the hyperactive Drp1 of HD cells impinges on cristae shape.

Discussion

The role of mitochondria in the pathogenesis of neurodegenerative diseases is under close scrutiny (Chan, 2007). This is not surprising, given their position at the crossroad of energy conversion and integration of apoptotic signalling. Mounting evidence support a role for altered mitochondrial shape in neurodegeneration: some neurodegenerative genetic disorders are for example directly linked to mutations in the genes that code for core components of the mitochondria-shaping machinery. In the case of Parkinson's disease, the genes mutated in the genetic familial form of the disorder have been reported to impinge on mitochondrial shape (Poole et al, 2008). Whether this is the case also for HD is less clear. Our data show that mitochondria in cells from HD patients or from a mouse model expressing a pathologic polyQ repeat are fragmented and display changes in the ultrastructure that are causally linked to the increased susceptibility to apoptosis.

Fission is one of the hallmarks of mitochondrial apoptosis, together with the remodelling of the cristae (Frank et al, 2001; Scorrano et al, 2002). Ectopic expression of a 74 polyQ from Htt in HeLa cells causes mitochondrial fragmentation. Based on pull-down experiments, Wang et al suggest that fragmentation could result from inhibition of Mfn2 (Wang et al, 2009). However, Mfns have two coiled coil regions which are prone to protein-protein interaction (de Brito & Scorrano, 2008a), thereby diminishing the confidence that such an interaction is relevant for the observed fragmentation. Evidence of cristae remodelling is one of the hallmarks of lymphoblasts from HD patients (Mormone et al, 2006), a widely employed model to study the mitochondrial alterations in the disease (Sawa et al, 1999; Panov et al, 2002). Our data show mitochondrial fragmentation in HD lymphoblasts in a Q-repeat and gene dosage dependent fashion, a result confirmed in striatal precursors and differentiated striatal neurons from the Q111 knock-in mouse (Trettel et al, 2000). Fragmentation associated with increased translocation and activation of Drp1 on mitochondria, a process that we recently found to be triggered by Drp1 dephosphorylation by the cytosolic phosphatase calcineurin (Cereghetti et al, 2008).

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Calcineurin received considerable attention in the context of HD. Its inhibition by FK506 protects from Htt induced toxicity (Xifro et al, 2008) and this has been linked to changes in Htt phosphorylation (Pardo et al, 2006). Moreover, levels of the calcineurin inhibitor RCAN1-1L are reduced in HD (Ermak et al, 2009) and the dysregulation of cytosolic Ca^{2+}, the proximal activator of calcineurin, is one of the hallmarks of HD (Tang et al, 2005). Our results suggest that another target of calcineurin in the context of HD can be Drp1 and hence mitochondrial morphology. In fact mitochondrial fragmentation can be reverted by genetic or pharmacological inhibition of calcineurin. It will be interesting to verify if the natural history of the disease would be modified in mouse models crossed with conditional models of Drp1 ablation recently described (Ishihara et al, 2009).

How does increased fragmentation of the mitochondria contribute to the progression of the apoptotic cascade? One unifying model suggests that activation of the pro-fission protein Drp1 not only causes the fragmentation of the organelle, but also induces remodelling of the cristae, thereby augmenting the availability of cytochrome *c* in the intermembrane space of the organelle and hence its release across the outer membrane (Germain et al, 2005). This was reinforced by recent data showing changes in Opa1 pattern in cells where expression of Drp1 was modulated (Mopert et al, 2009). Such a scenario seems plausible also in the HD models tested here. The fragmented mitochondria of HD lymphoblasts and striatal precursors are more susceptible to cytochrome *c* release and oligomers of Opa1, which control cristae junctions (Cipolat et al, 2006; Frezza et al, 2006; Yamaguchi et al, 2008) are readily destabilized in the HD cells. Accordingly, we were able to maintain the integrity of mitochondrial cristae during apoptosis not only by the enforced expression of Opa1, but also by a dominant negative mutant of Drp1. Both Opa1 and mutant Drp1 protect the HD models from cell death, while enforcing fusion by expression of Mfn1 does not. Notably, Mfn1 does not affect shape of the cristae and is dispensable for the antiapoptotic activity of Opa1 (Frezza et al, 2006). This further substantiates the role of ultrastructural changes in the HD phenotype analyzed here.

How mitochondria are affected in HD is a matter of debate. It has been reported that the multiple changes in mitochondrial Ca^{2+} handling (Panov et al, 2002), metabolism (Damiano et al, 2010), susceptibility to apoptosis (Sawa et al, 1999) could be related to mitochondrial localization of mutated Htt (Orr et al, 2008) or to transcriptional regulation of the master mitochondrial biogenetic gene PGC1α (Cui et al, 2006). Here we add one potential mechanism of structural alterations, orchestrated by calcineurin-dependent mitochondrial translocation and activation of the pro-fission protein Drp1. Our studies lend the molecular basis for future in vivo studies to verify the contribution of this pathway to the neurological phenotype of the disease.

Materials and Methods

Plasmids

mtYFP, pCB6-MYC-Mfn1, pcDNA3.1-HA-K38A-DRP1, pMSCV-OPA1, pEGFP were described in (Cipolat et al, 2004; de Brito & Scorrano, 2008b). pcDNA3.1-H155Q-CnA was described in (Cereghetti et al, 2008).

Cell Culture, Transfection, Reagents and Sorting

EBV immortalized human B lymphoblasts from one unaffected voluntary subject and one heterozygous HD patient with 48 CAG repeats were described in (Mormone et al., 2006). Lymphoblasts from two male subjects (control and homozygous with 45+47 CAG repeats) and two female subjects (control and heterozygous with 70 CAG repeats) were obtained from the Coriell Institute for Medical Research. Lymphoblasts were cultured in DMEM-F12 (Gibco), 20% fetal bovine serum (FBS), 50 U/mL Penicillin, 50 μg/mL Streptomycin, 100 μM non essential amino acids (MEM, Gibco/Invitrogen) and 2 mM glutamine (Gibco). Lymphoblasts were electroporated using the MicroPorator system (Digital Bio Technology) following manufacturer's instructions.

Clonal striatal cell lines established from E14 striatal primordial of KI-Hdh Q111 and WT-Hdh Q7 littermate mouse embryos were described previously (Trettel et al., 2000). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), 10% fetal bovine serum (FBS), 50 U/mL Penicillin, 50 μg/mL Streptomycin, 100 μM non essential amino acids (MEM, Gibco/Invitrogen) and 2 mM glutamine (Gibco). They were maintained at the permissive temperature of 33°C. Neurons were transfected using Transfectin Lipid Reagent (Biorad) following manufacturer's instructions.

For sorting, 4×10^6 cotransfected neurons were analyzed by light forward and side scatter and for GFP fluorescence through a 530 nm band pass filter as they traversed the beam of an argon ion laser (488

nm, 100 mW) of a FACSAria (BD). Non transfected neurons were used to set the background fluorescence.

Analysis of Cell Death

Lymphoblasts treated as indicated were stained with propidium iodide (PI) and annexin-V-FITC (Bender MedSystem). Where indicated, cells were cotransfected with pEGFP and the indicated vector. After 24 hr cells were treated as described and stained with Annexin-V-PE (Bender MedSystem) according to the manufacturer's protocol. Cell death was measured by flow cytometry (FACSCalibur) as the percentage of Annexin-V-positive events in the GFP-positive population and viability as the percentage of Annexin-V-negative, PI-negative cells for transfected and untransfected cells respectively.

In Vitro Mitochondrial Assays

Mitochondria were isolated by standard differential centrifugation in isolation buffer (IB) as described in (Frezza et al, 2007). Cytochrome c release in response to recombinant cBID was determined as described in (Frezza et al, 2006). p7/p15 recombinant BID was produced, purified, and cleaved with caspase-8 as described in (Frezza et al, 2006). Unless noted, it was used at a final concentration of 32 pmol x mg $^{-1}$.

Biochemistry

For protein crosslinking, mitochondria were treated with 2 mM EDC (Pierce) in EB for 30 min at 37°C. Samples were centrifuged for 10 min at 12000×g at 4°C, and the mitochondrial pellets were resuspended in SDS-PAGE sample loading buffer. DTT in the sample buffer quenched the crosslinking reaction.

For DRP1 crosslinking studies, cells were lysed in 6mM CHAPS in PBS for 1 hr at 4°C; an equal amount of proteins from each lysate was treated with 1 mM BS^3 (Pierce) 2 hrs on ice. The reaction was stopped by 15 min incubation with 100 mM Tris/HCl buffer (pH 7.4) at 25°C.

For OPA1 crosslinking experiments in neurons, cells were treated as indicated and incubated with 1 mM DSS (Pierce) for 30 min at 25°C. The reaction was quenched by 15 min incubation with 100 mM Tris/HCl buffer (pH 7.4) at 25°C.

For immunoblotting, proteins were separated by 7% Tris-Acetate, 3%-8% Tris-Acetate or 4%-12% Tris-MOPS SDS-PAGE (NuPage, Invitrogen), transferred onto PVDF membranes (Millipore), and probed using the indicated primary antibodies and isotype matched secondary antibodies conjugated to horseradish peroxidase. Signals were detected using ECL (Amersham).

Antibodies

For immunoblotting experiments the following antibodies were employed: mouse monoclonal anti-OPA1 antibody (1:1500, BD Biosciences); mouse monoclonal anti-DRP1 (1:1500, BD Biosciences); mouse monoclonal anti-MFN2 (1:1000, Abnova); rabbit polyclonal anti-FIS1 (1:1000 Abnova); rabbit polyclonal anti-TOM20 (1:2000, St. Cruz Biotecnology); mouse monoclonal anti-actin (1:3000, Chemicon); rabbit polyclonal anti-PARP (1:1000, Cell Signaling). Isotype matched, horseradish peroxidase conjugated secondary antibodies (Amersham) were used followed by detection by chemiluminescence (Amersham).

Trasmission Electron Microscopy and Mitochondrial Morphometry

Cells were fixed for 1 hr at 25°C using glutaraldehyde at a final concentration of 2.5% (v/v) in PBS. Embedding and staining were performed as described in (Scorrano et al, 2002). Thin sections were

imaged on a Tecnai-20 electron microscope (Philips-FEI). Mitochondrial cristae morphology was analyzed using MetaMorph software.

Imaging

For imaging of mitochondrial network, lymphoblasts of the indicated genotype were transfected as indicated. After 24 hrs, cells were plated in serum-free medium for 1.5 hrs to promote adhesion to fibronectin (5 µg/ml, Sigma) coated coverslips. Samples were analyzed with a Nikon Eclipse TE300 inverted microscope equipped with a Perkin Elmer Ultraview LCI. A piezoelectric axis motorized stage (Pifoc, Physik Instrumente), and an Orca ER 12-bit CCD camera (Hamamatsu Photonics). Cells expressing mtYFP were excited using the 488 nm line of the He-Ne laser (Perkin Elmer) with exposure times of 100 msec using a 100x1.4 NA Plan Apo objective (Nikon). Stacks separated by 0.4 µm along the z axis were acquired. 3D reconstruction and volume rendering were performed using a plug-in of ImageJ (NIH).

For confocal imaging of neurons, 10^5 cells were seeded onto 24 mm-round glass coverslips and transfected as indicated. After 24 hrs cells were incubated in Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM Hepes and coverslips were placed on the stage of a Zeiss LSM 510 inverted microscope. Cells expressing mtYFP were excited using the 488 nm line of the Argon laser using a 63x1.4 NA Plan Apochromat objective (Zeiss).

Quantitative analysis of mitochondria morphology was performed as previously described (Cipolat et al, 2004). Cells were classified as having fragmented mitochondria when more than 50% of the total cellular organelles displayed a major axis shorter than 5 µm for neurons and 3 µm for lymphoblasts.

For immunofluorescence experiments, 12×10^4 cells were plated onto 24 mm-round glass coverslips and transfected with mtRFP. After 24 hrs cells were treated for 2 hrs with 1 mM H_2O_2 in complete medium, fixed and immunostained with anti-cytochrome *c* antibody (1:200, BD Biosciences) and isotype

matched FITC-conjugated secondary antibody. For cytochrome c and mtRFP detection green and red channel images were acquired simultaneously using two separate color channels on the detector assembly of a Zeiss LSM 510 using a 63x1.4 NA Plan Apochromat objective (Zeiss). The localization index was calculated as described in (Frezza et al., 2006).

Acknowledgements

References

Alexander C et al (2000) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet 26: 211-215

Almeida S, Domingues A, Rodrigues L, Oliveira CR, Rego AC (2004) FK506 prevents mitochondrial-dependent apoptotic cell death induced by 3-nitropropionic acid in rat primary cortical cultures. Neurobiol Dis 17: 435-444

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT (1993) Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci 13: 4181-4192

Cereghetti GM, Stangherlin A, Martins de BO, Chang CR, Blackstone C, Bernardi P, Scorrano L (2008) Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc Natl Acad Sci U S A 105: 15803-15808

Chan DC (2006) Mitochondria: dynamic organelles in disease, aging, and development. Cell 125: 1241-1252

Chan DC (2007) Mitochondrial dynamics in disease. N Engl J Med 356: 1707-1709

Chen H, McCaffery JM, Chan DC (2007) Mitochondrial Fusion Protects against Neurodegeneration in the Cerebellum. Cell 130: 548-562

Choo YS, Johnson GV, MacDonald M, Detloff PJ, Lesort M (2004) Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. Hum Mol Genet 13: 1407-1420

Cipolat S, de Brito OM, Dal Zilio B, Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A 101: 15927-15932

Cipolat S et al (2006) Mitochondrial Rhomboid PARL Regulates Cytochrome c Release during Apoptosis via OPA1-Dependent Cristae Remodeling. Cell 126: 163-175

Cui L, Jeong H, Borovecki F, Parkhurst CN, Tanese N, Krainc D (2006) Transcriptional Repression of PGC-1[alpha] by Mutant Huntingtin Leads to Mitochondrial Dysfunction and Neurodegeneration. Cell 127: 59-69

Damiano M, Galvan L, Déglon N, Brouillet E (2010) Mitochondria in Huntington's disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1802: 52-61

Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116: 205-219

de Brito OM, Scorrano L (2008a) Mitofusin 2: a mitochondria-shaping protein with signaling roles beyond fusion. Antioxid Redox Signal 10: 621-633

de Brito OM, Scorrano L (2008b) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 456: 605-610

De Rooij KE, Dorsman JC, Smoor MA, Den Dunnen JT, van Ommen GJ (1996) Subcellular localization of the Huntington's disease gene product in cell lines by immunofluorescence and biochemical subcellular fractionation. Hum Mol Genet 5: 1093-1099

Delettre C et al (2000) Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat Genet 26: 207-210

DeVay RM, Dominguez-Ramirez L, Lackner LL, Hoppins S, Stahlberg H, Nunnari J (2009) Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. J Cell Biol 186: 793-803

Difiglia M et al (1995) Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. Neuron 14: 1075-1081

Difiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277: 1990-1993

Duyao M et al (1993) Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat Genet 4: 387-392

Ermak G, Hench KJ, Chang KT, Sachdev S, Davies KJ (2009) Regulator of calcineurin (RCAN1-1L) is deficient in Huntington disease and protective against mutant huntingtin toxicity in vitro. J Biol Chem 284: 11845-11853

Fernandes HB, Baimbridge KG, Church J, Hayden MR, Raymond LA (2007) Mitochondrial sensitivity and altered calcium handling underlie enhanced NMDA-induced apoptosis in YAC128 model of Huntington's disease. J Neurosci 27: 13614-13623

Ferrante RJ, Kowall NW, Richardson EP, Jr. (1991) Proliferative and degenerative changes in striatal spiny neurons in Huntington's disease: a combined study using the section-Golgi method and calbindin D28k immunocytochemistry. J Neurosci 11: 3877-3887

Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1: 515-525

Frezza C et al (2006) OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion. Cell 126: 177-189

Frezza C, Cipolat S, Scorrano L (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2: 287-295

Gautier CA, Kitada T, Shen J (2008) Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc Natl Acad Sci U S A 105: 11364-11369

Germain M, Mathai JP, McBride HM, Shore GC (2005) Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. EMBO J 24: 1546-1556

Graham RK et al (2006) Cleavage at the caspase-6 site is required for neuronal dysfunction and degeneration due to mutant huntingtin. Cell 125: 1179-1191

Grunewald T, Beal MF (1999) Bioenergetics in Huntington's disease. Ann N Y Acad Sci 893: 203-213

Gu X, Greiner ER, Mishra R, Kodali R, Osmand A, Finkbeiner S, Steffan JS, Thompson LM, Wetzel R, Yang XW (2009) Serines 13 and 16 are critical determinants of full-length human mutant huntingtin induced disease pathogenesis in HD mice. Neuron 64: 828-840

Gunawardena S, Her LS, Brusch RG, Laymon RA, Niesman IR, Gordesky-Gold B, Sintasath L, Bonini NM, Goldstein LS (2003) Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila. Neuron 40: 25-40

Gutekunst CA, Levey AI, Heilman CJ, Whaley WL, Yi H, Nash NR, Rees HD, Madden JJ, Hersch SM (1995) Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. Proc Natl Acad Sci U S A 92: 8710-8714

Hackam AS, Singaraja R, Wellington CL, Metzler M, McCutcheon K, Zhang T, Kalchman M, Hayden MR (1998) The influence of huntingtin protein size on nuclear localization and cellular toxicity. J Cell Biol 141: 1097-1105

Ishihara N et al (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol 11: 958-966

Jana NR, Zemskov EA, Wang G, Nukina N (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum Mol Genet 10: 1049-1059

Kegel KB et al (2002) Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. J Biol Chem 277: 7466-7476

Li Z, Okamoto K, Hayashi Y, Sheng M (2004) The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell 119: 873-887

Liesa M, Palacin M, Zorzano A (2009) Mitochondrial dynamics in mammalian health and disease. Physiol Rev 89: 799-845

Liot G, Bossy B, Lubitz S, Kushnareva Y, Sejbuk N, Bossy-Wetzel E (2009) Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death. Cell Death Differ 16: 899-909

Lutz AK et al (2009) Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. J Biol Chem 284: 22938-22951

Martin JB, Gusella JF (1986) Huntington's disease. Pathogenesis and management. N Engl J Med 315: 1267-1276

Mopert K, Hajek P, Frank S, Chen C, Kaufmann J, Santel A (2009) Loss of Drp1 function alters OPA1 processing and changes mitochondrial membrane organization. Exp Cell Res 315: 2165-2180

Morais VA et al (2009) Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. EMBO Mol Med 1: 1-14

Mormone E, Matarrese P, Tinari A, Cannella M, Maglione V, Farrace MG, Piacentini M, Frati L, Malorni W, Squitieri F (2006) Genotype-dependent priming to self- and xeno-cannibalism in heterozygous and homozygous lymphoblasts from patients with Huntington's disease. J Neurochem 98: 1090-1099

Niemann A, Ruegg M, La P, V, Schenone A, Suter U (2005) Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot-Marie-Tooth disease. J Cell Biol 170: 1067-1078

Orr AL, Li S, Wang CE, Li H, Wang J, Rong J, Xu X, Mastroberardino PG, Greenamyre JT, Li XJ (2008) N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. J Neurosci 28: 2783-2792

Panov AV, Gutekunst CA, Leavitt BR, Hayden MR, Burke JR, Strittmatter WJ, Greenamyre JT (2002) Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat Neurosci 5: 731-736

Pardo R, Colin E, Regulier E, Aebischer P, Deglon N, Humbert S, Saudou F (2006) Inhibition of calcineurin by FK506 protects against polyglutamine-huntingtin toxicity through an increase of huntingtin phosphorylation at S421. J Neurosci 26: 1635-1645

Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ (2008) The PINK1/Parkin pathway regulates mitochondrial morphology. Proc Natl Acad Sci U S A 105: 1638-1643

Ravikumar B et al (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 36: 585-595

Sawa A, Wiegand GW, Cooper J, Margolis RL, Sharp AH, Lawler JF, Jr., Greenamyre JT, Snyder SH, Ross CA (1999) Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. Nat Med 5: 1194-1198

Schilling G, Sharp AH, Loev SJ, Wagster MV, Li SH, Stine OC, Ross CA (1995) Expression of the Huntington's disease (IT15) protein product in HD patients. Hum Mol Genet 4: 1365-1371

Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ (2002) A Distinct Pathway Remodels Mitochondrial Cristae and Mobilizes Cytochrome c during Apoptosis. Dev Cell 2: 55-67

Sharp AH et al (1995) Widespread expression of Huntington's disease gene (IT15) protein product. Neuron 14: 1065-1074

Sugars KL, Rubinsztein DC (2003) Transcriptional abnormalities in Huntington disease. Trends Genet 19: 233-238

Tang TS, Slow E, Lupu V, Stavrovskaya IG, Sugimori M, Llin+is R, Kristal BS, Hayden MR, Bezprozvanny I (2005) Disturbed Ca2+ signaling and apoptosis of medium spiny neurons in Huntington's disease. Proceedings of the National Academy of Sciences of the United States of America 102: 2602-2607

The Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell 72: 971-983

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456-1462

Trettel F, Rigamonti D, Hilditch-Maguire P, Wheeler VC, Sharp AH, Persichetti F, Cattaneo E, MacDonald ME (2000) Dominant phenotypes produced by the HD mutation in STHdh(Q111) striatal cells. Hum Mol Genet 9: 2799-2809

Trushina E et al (2004) Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. Mol Cell Biol 24: 8195-8209

Wang H, Lim PJ, Karbowski M, Monteiro MJ (2009) Effects of overexpression of huntingtin proteins on mitochondrial integrity. Hum Mol Genet 18: 737-752

Wasilewski M, Scorrano L (2009) The changing shape of mitochondrial apoptosis. Trends Endocrinol Metab 20: 287-294

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292: 727-730

Wellington CL et al (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. J Biol Chem 275: 19831-19838

Xifro X, Garcia-Martinez JM, del TD, Alberch J, Perez-Navarro E (2008) Calcineurin is involved in the early activation of NMDA-mediated cell death in mutant huntingtin knock-in striatal cells. J Neurochem 105: 1596-1612

Yamaguchi R, Lartigue L, Perkins G, Scott RT, Dixit A, Kushnareva Y, Kuwana T, Ellisman MH, Newmeyer DD (2008) Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak oligomerization. Mol Cell 31: 557-569

Zeitlin S, Liu JP, Chapman DL, Papaioannou VE, Efstratiadis A (1995) Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. Nat Genet 11: 155-163

Zeron MM, Hansson O, Chen N, Wellington CL, Leavitt BR, Brundin P, Hayden MR, Raymond LA (2002) Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33: 849-860

Zhu PP, Patterson A, Stadler J, Seeburg DP, Sheng M, Blackstone C (2004) Intra- and intermolecular domain interactions of the C-terminal GTPase effector domain of the multimeric dynamin-like GTPase Drp1. J Biol Chem 279: 35967-35974

Zuchner S et al (2004) Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat Genet 36: 449-451

Figure legends

Figure 1. **Mitochondrial fragmentation and cristae derangement in HD lymphoblasts and neurons.**

(A) Lymphoblasts of the indicated genotype were transfected with mtYFP. Randomly selected confocal, 14 μm deep z axis stacks were acquired, stored, reconstructed, and volume rendered. Scale bar, 5μm.

(B) Morphometric analysis of mitochondrial shape. Experiments were as in (A). Thirty randomly selected, reconstructed and volume rendered z stack series were classified as described. Wt refers to gender-matched control of HD lymphoblasts. Data represent mean ± SE of four independent experiments.

(C) Neurons of the indicated genotype were transfected with mtYFP. Confocal images of mtYFP from randomly selected cells. Scale bar, 20 μm.

(D) Morphometric analysis of mitochondrial morphology. Experiments were as in (B). Fifty randomly selected images of mtYFP fluorescence were acquired, stored and classified as described. Data represent mean ± SE of five independent experiments.

(E) Representative electron micrographs of wt and HD (Q111) striatal neurons. Cells were fixed and TEM images of randomly selected fields were acquired. Boxed areas represent a 2.3× magnification. Scale bar, 1μm.

Figure 2. **Translocation and activation of Drp1 in HD cells.**

(A and B) Equal amounts of proteins (20 μg) from lymphoblasts (A) and striatal precursors (B) of the indicated genotype were separated by SDS-PAGE and immunoblotted with the indicated antibodies. For lymphoblasts, Wt refers to gender-matched control.

(C and D) Equal amounts of protein (30 μg) from mitochondria isolated from neurons (C) and lymphoblasts (D) of the indicated genotype were analyzed by SDS-PAGE/immunoblotting using the indicated antibodies.

(E-G) Cell lysates from lymphoblasts (E and F) and neurons (G) of the indicated genotype treated as indicated for 2 hrs. The reaction was quenched and equal amounts of proteins (20 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

Figure 3. Correction of mitochondrial morphology in HD cells.

(A,C) Lymphoblasts of the indicated genotype were cotransfected with mtYFP and empty vector or the indicated plasmids. Experiments were performed exactly as in Fig. 1. Scale bar, 5 μm.

(B,D) Morphometric analysis of mitochondrial shape. Experiments were as in A and C, respectively. Thirty randomly selected, reconstructed and volume rendered z stack series were classified as described. Data represent mean ± SE of four independent experiments.

(E) Cells of the indicated genotype were cotransfected with mtYFP and empty vector or the indicated plasmids. When indicated, cells were treated with 1 μM FK506 for 1 hr before acquisition of images. Images were acquired exactly as in Fig. 1. Scale bar, 20 μm.

(F) Morphometric analysis of mitochondrial morphology. Experiments were as in E. Fifty randomly selected images of mtYFP fluorescence were acquired, stored and classified as described. Data represent mean ± SE of five independent experiments.

Figure 4. HD cells are more susceptible to apoptosis.

(A) Lymphoblasts of the indicated genotype were treated with 2 μM staurosporine. At the indicated times, viability was determined cytofluorimetrically as the percentage of Annexin-V-negative, PI-negative cells. Data represent mean ± SE of seven independent experiments.

(B) Neurons of the indicated genotype were treated with 2 μM staurosporine. At the indicated times, cells were lysed and equal amounts of proteins (30 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

Figure 5. Cytochrome *c* release and disruption of OPA1 oligomers is faster in HD cells.

(A) Representative confocal images of striatal cells of the indicated genotype transfected with mtRFP and immunostained with anti-cytochrome *c* (green) antibody. When indicated, cells were treated for 2 hrs with 1mM H_2O_2.

(B) Localization index of cytochrome *c*. Cells of the indicated genotype were treated where indicated for 2 hrs with 1mM H_2O_2 or for 5 hrs with 0.75 μM staurosporine (STS). Localization index of cytochrome *c* was determined as described (Frezza et al, 2006). Data represent mean ±SE of 3 independent experiments (n=50 randomly selected cells per condition in each experiment).

(C, E, G) Mitochondria isolated from lymphoblasts of the indicated genotype were treated for the indicated times with cBID. After centrifugation the amount of cytochrome *c* in supernatant and pellet was determined by a specific ELISA. Data represent mean ± SE of four independent experiments.

(D, F, H) Mitochondria from lymphoblasts of the indicated genotype were treated with cBID for the indicated times and then crosslinked with 2 mM EDC. After 30 min the reaction was quenched and equal amounts of proteins were analyzed by SDS-PAGE/immunoblotting using anti-OPA1 antibody.

Figure 6. Apoptotic ultrastructural changes in HD mitochondria are faster and prevented by OPA1 and blockage of DRP1.

(A) Representative EM fields of neurons of the indicated genotype. Cells were cotransfected with GFP and the indicated plasmids and then sorted by cytofluorimetry. GFP-positive cells were seeded and after 18 hrs cells were treated when indicated with 2 μM staurosporine for 3 hrs. Scale bar, 1 μm.

(B) Morphometric analysis of randomly selected EM fields of mitochondria from neurons of the indicated genotype. Experiments were performed as in A. n=50 mitochondria per sample.

Figure 7. Correction of apoptosis in HD cells by enforced expression of Opa1 or blockage of Drp1.
(A) Lymphoblasts of the indicated genotype transfected as indicated were treated with 2 μM staurosporine. At the indicated times, cell death was determined cytofluorimetrically as the percentage of Annexin-V-positive cells in the GFP-positive population. Wt refers to gender-matched control of HD lymphoblasts. Data represent mean ± SE of five independent experiments.

(B-D) Neurons of the indicated genotype were cotransfected with GFP and empty vector or the indicated vectors. After 24 hrs cells were treated with 2 μM staurosporine for 3 hrs and 3×10^5 GFP positive cells were sorted by cytofluorimetry and lysed. Proteins were analyzed by SDS-PAGE/immunoblotting using the indicated antibodies.

(E) Neurons of the indicated genotype were treated when indicated with 1μM FK506 for 5 hrs, 2 μM staurosporine for 3 hrs or with 1 μM FK506 for 2 hrs followed by a 3 hrs treatment with staurosporine. Cells were lysed and equal amounts of proteins (30 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.
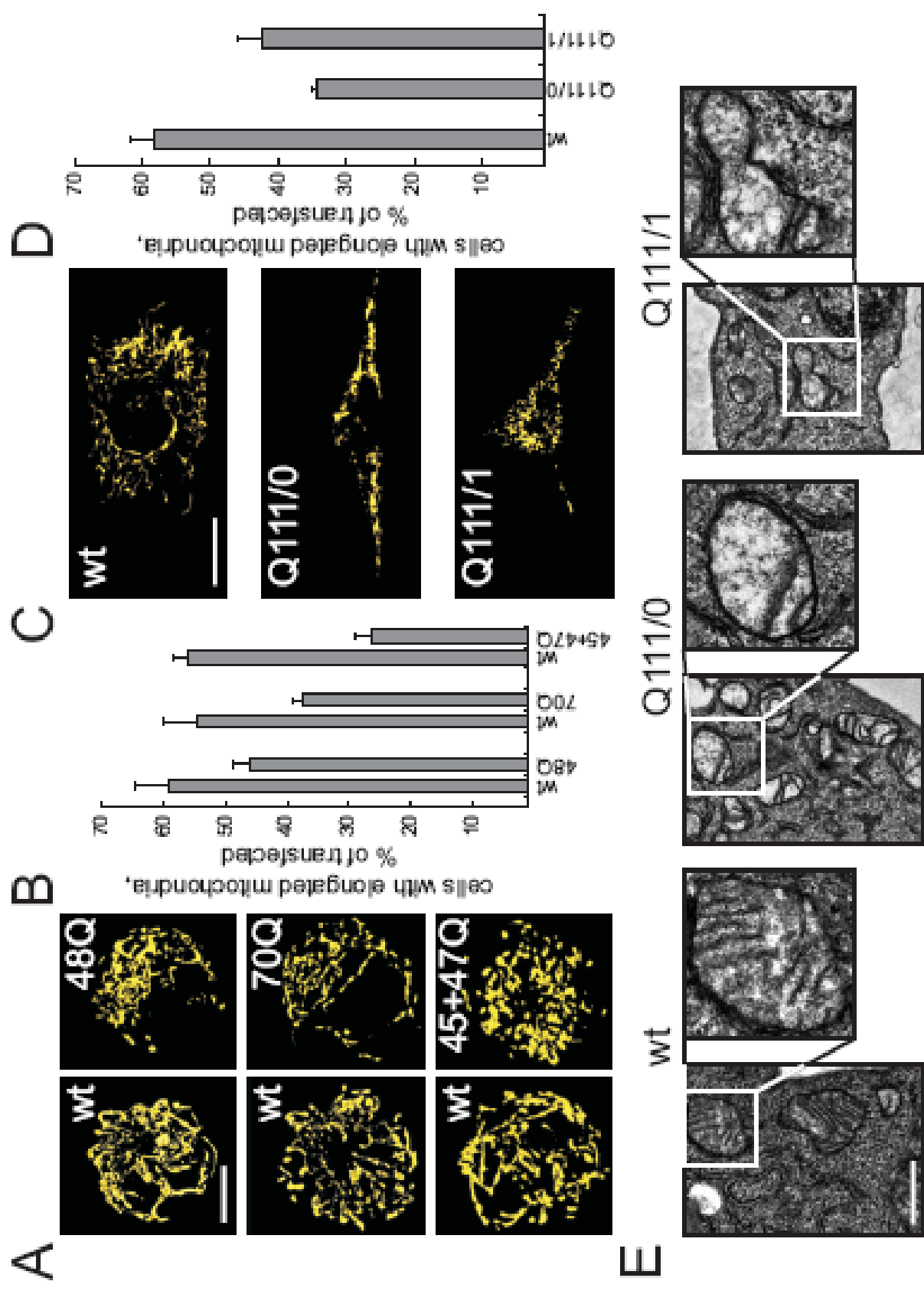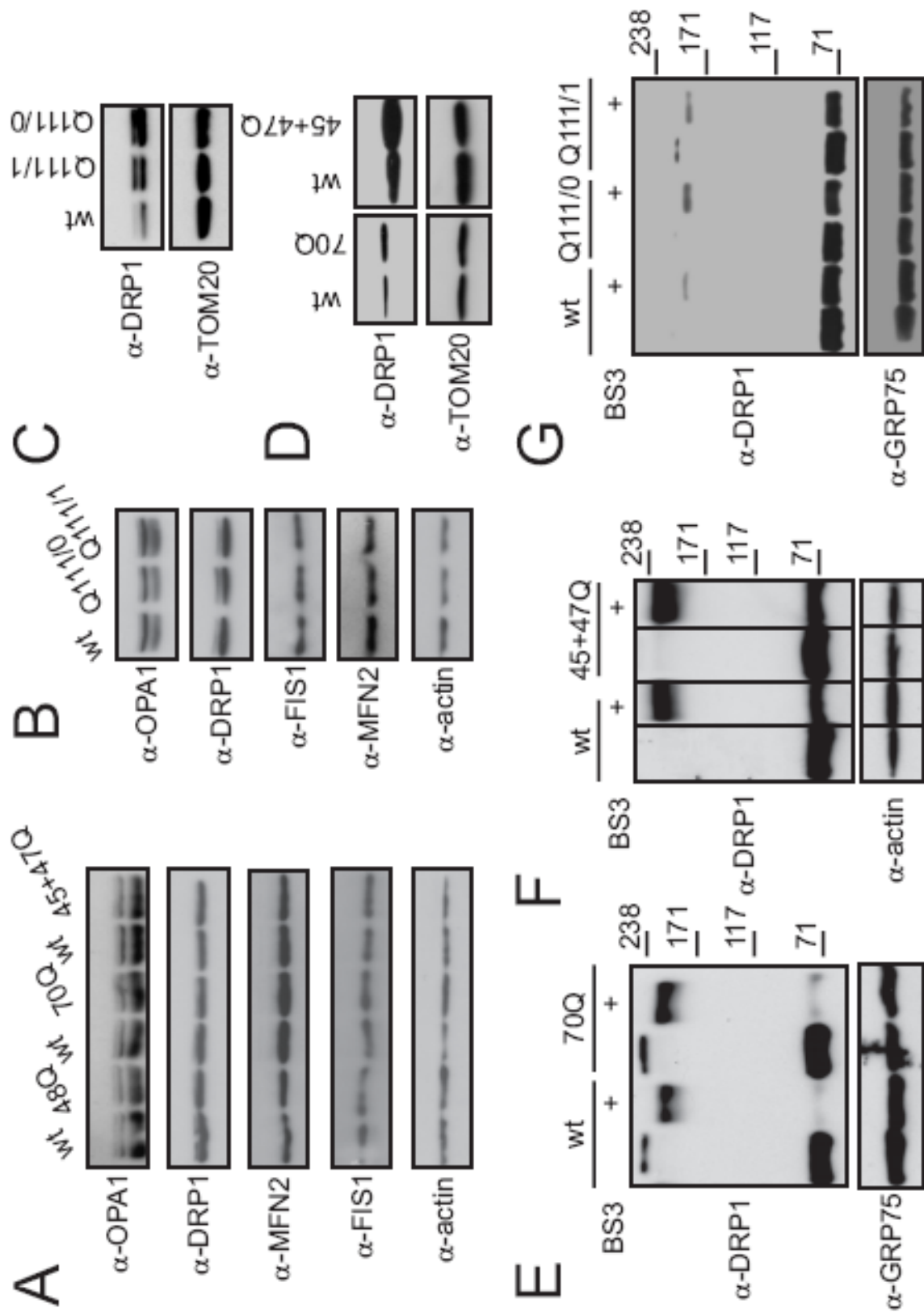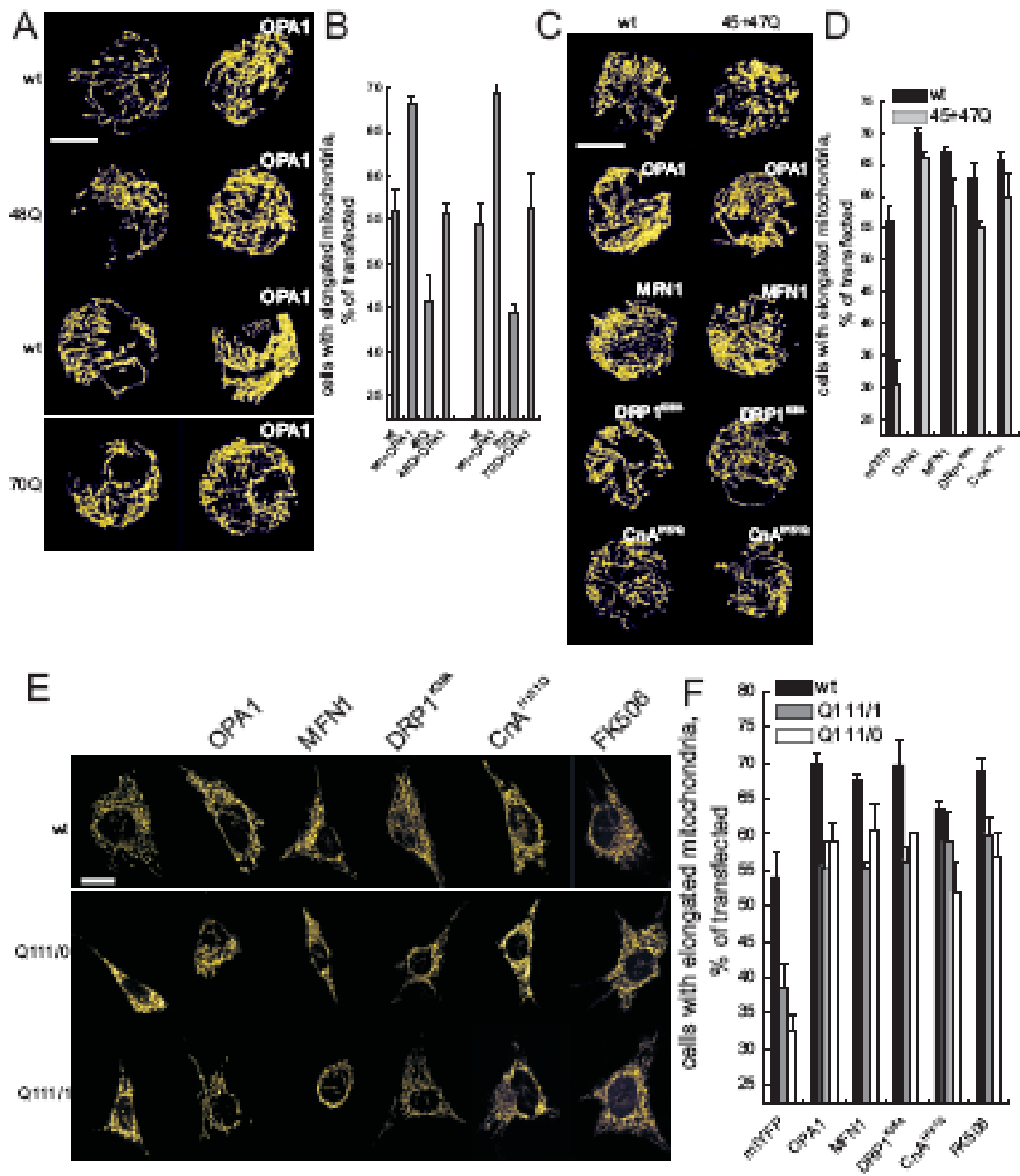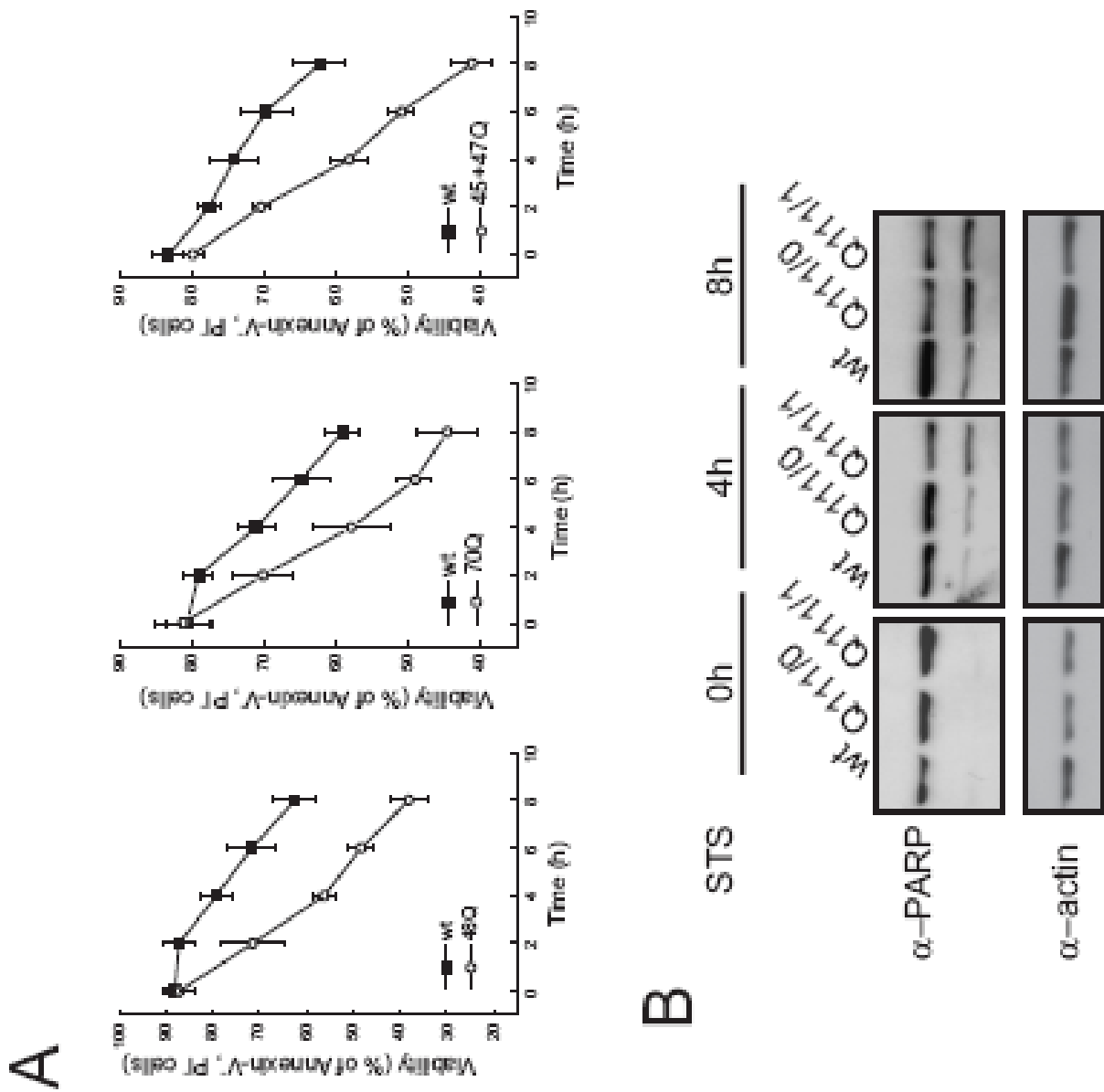
Figure 1

Figure 2

Figure 3

Figure 4
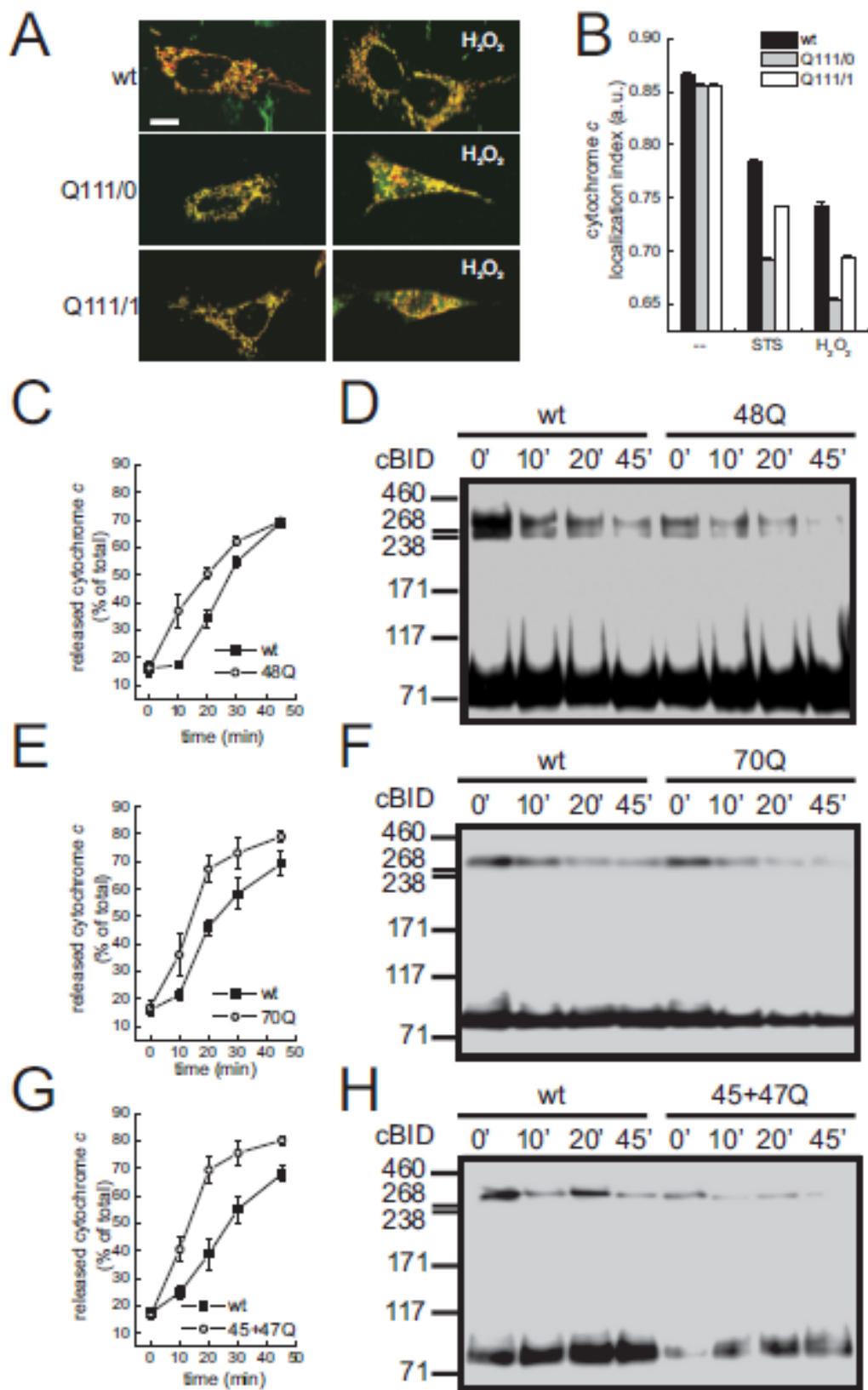
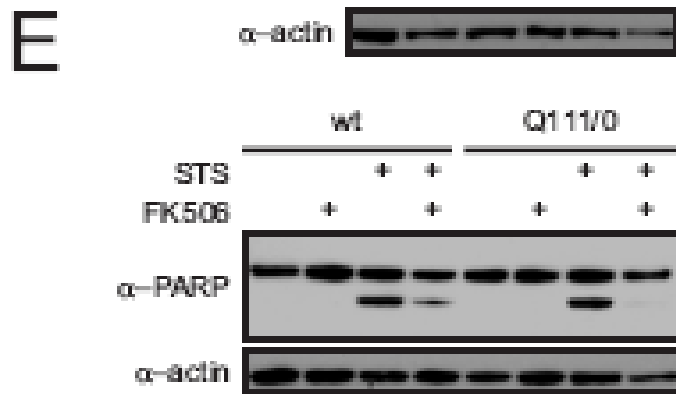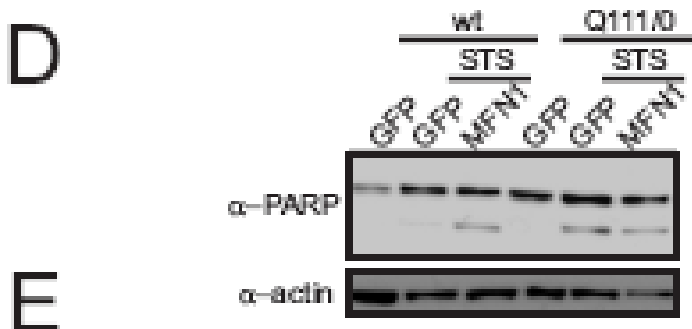**Figure 5**
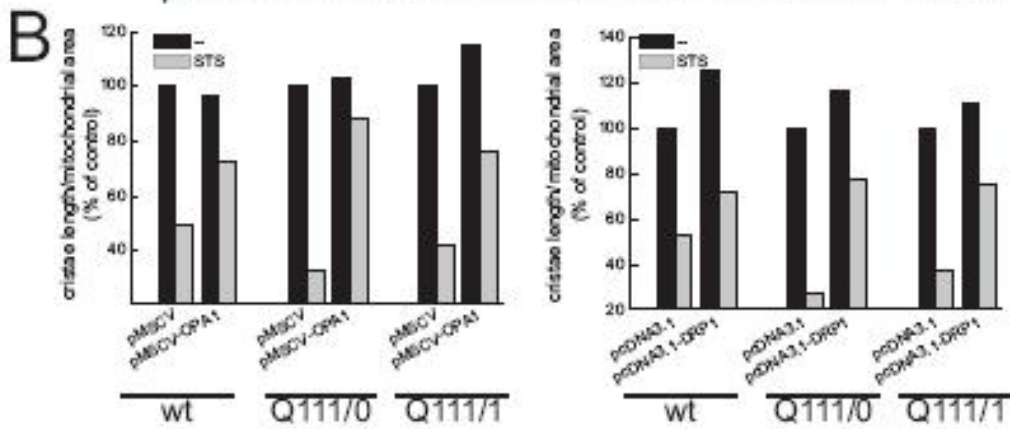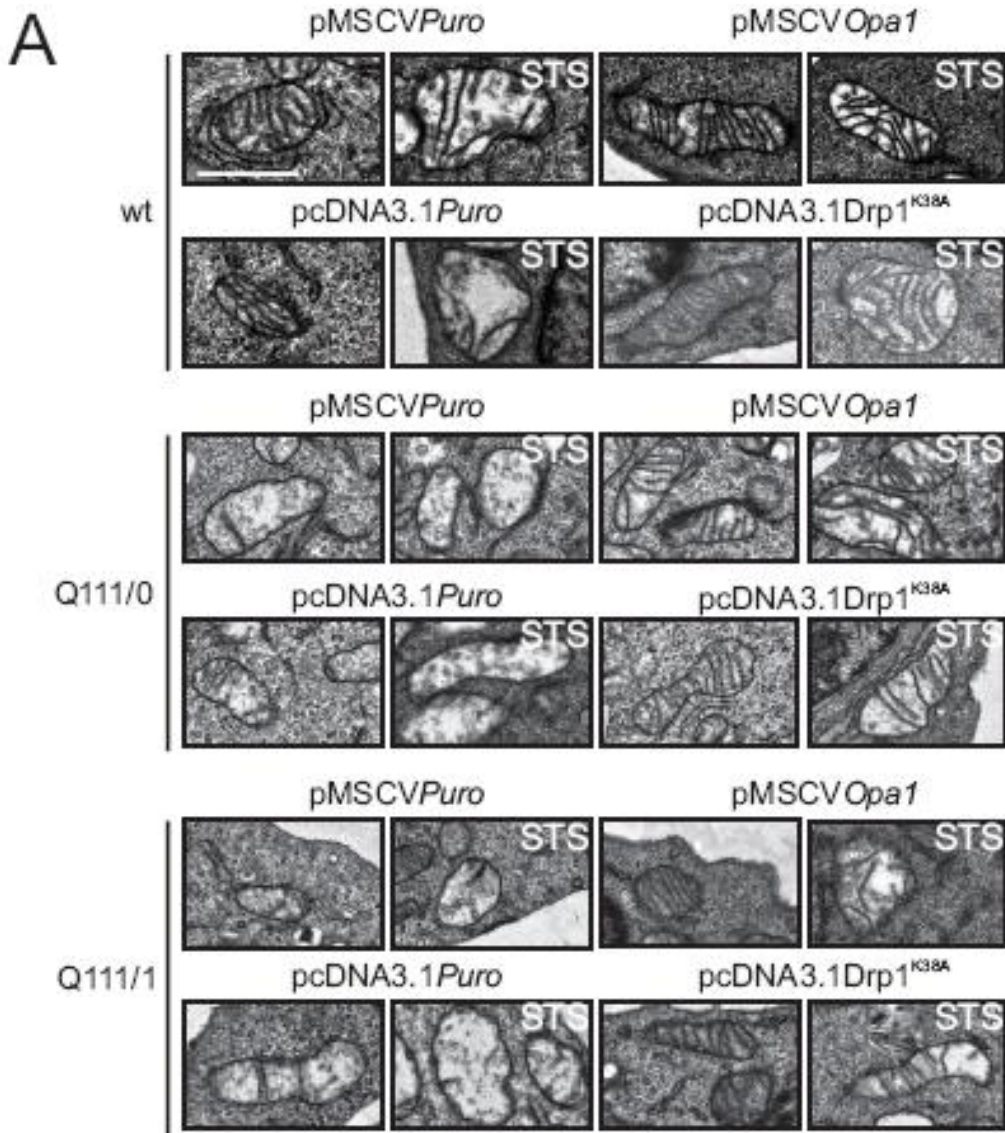
Figure 6
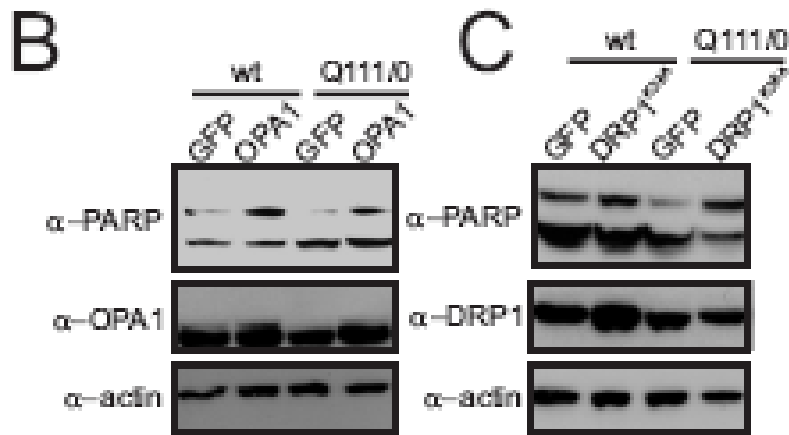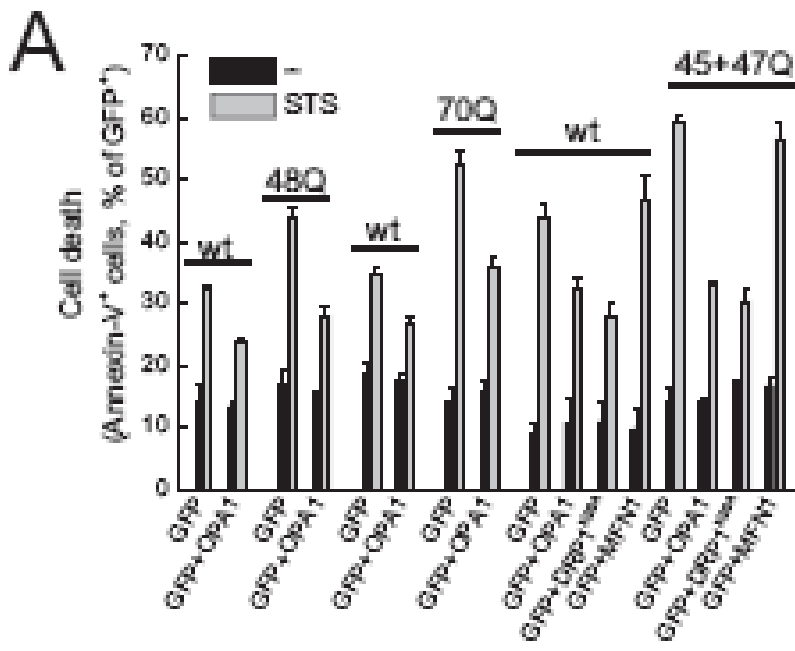
Figure 7

Supplementary Materials and Methods

Neuronal Differentiation and Imaging

Neuronal differentiation was induced as described in (Trettel et al, 2000). Briefly, cells were incubated for 14 hrs in serum-free DMEM supplemented with α-FGF (10ng/ml) (Abcam), IBMX (240 μM), TPA (20 μM), forskolin (48.6 μM) and dopamine (5 μM) (Sigma).

For imaging of mitochondrial network cells were loaded with 20 nM TMRM (Sigma) in HBSS supplemented with 10 mM Hepes for 30 min at 37°C.

Legend to supplementary Figures

Figure S1. Mitochondrial morphology in striatal precursors following neuronal differentiation.
Confocal images of randomly selected differentiated neuron-like cells loaded with TMRM. Scale bar, 20 μm.

Figure S2. Apoptotic changes in Opa1 oligomerization and cleavage in striatal HD precursors.
(A) An equal number (5×10^5) of neurons of the indicated genotype was treated with 1 mM H_2O_2 for 6 hrs. Cells were then treated with 1 mM DSS and after 30 min the reaction was quenched. Equal amounts of proteins were analyzed by SDS-PAGE/immunoblotting using anti-OPA1 antibody.

(H) Neurons of the indicated genotype were treated when indicated with 2 μM staurosporine for 3 hrs. Equal amounts of proteins (20 μg) were analyzed by SDS-PAGE/immunoblotting using the indicated antibodies.
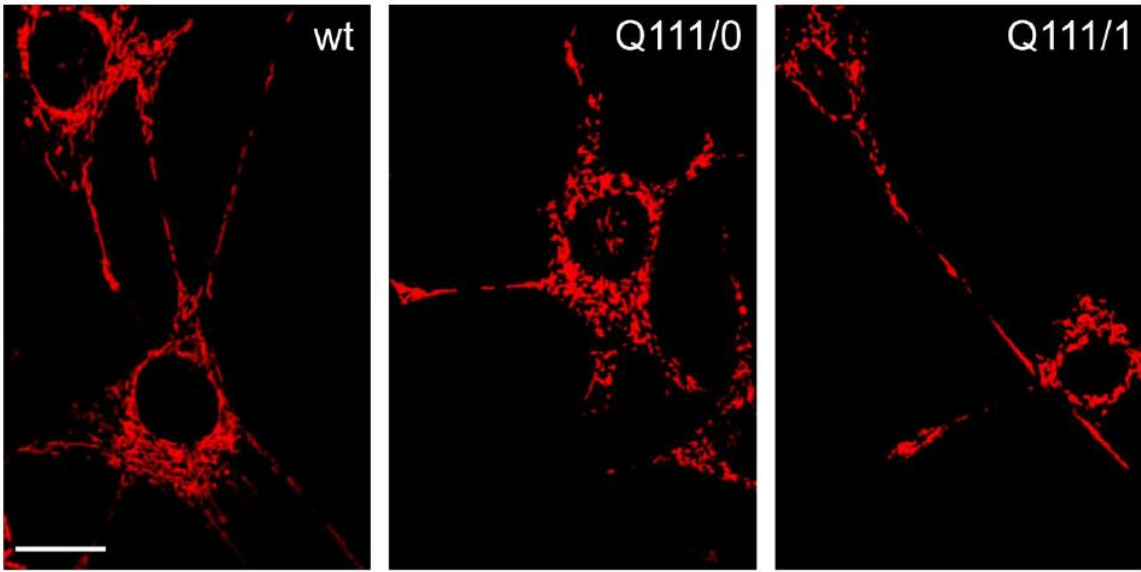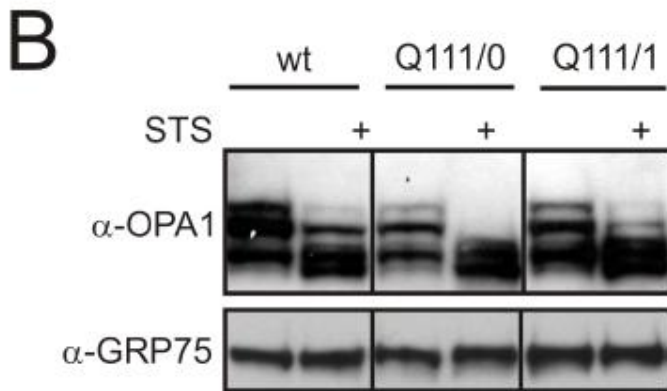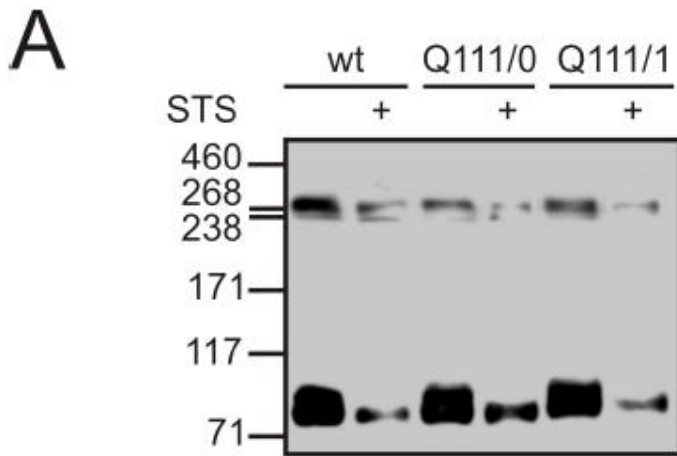
Figure S1

Figure S2

5 Conclusion and Perspectives

In this Thesis we analyzed the role of calcineurin-dependent mitochondrial fission in cell death and the relevance of this pathway in the pathogenesis of Hungtinton's disease.

Our results show that the specific inhibition of the phosphatase calcineurin by the peptide PPD1, corresponding to the peptidylprolyl isomerase domain of FKBP52, inhibits apoptosis delaying cytochrome c release. Notably, the protective role of calcineurin inhibition results from the block of the translocation of the pro-fission protein Drp1 to mitochondria and the subsequent inhibition of mitochondrial fragmentation during apoptosis. These data further support the view that Drp1 and mitochondrial fission participates in cell death.

Increasing evidence indicates that mitochondrial dynamics are essential for neuronal synaptic maintenance and plasticity and abnormal mitochondrial fragmentation is a hallmark of several neurodegenerative diseases associated with increased apoptosis. We showed that in Huntington's disease mitochondria are highly fragmented and this is due to the hyper-activation of the mitochondrial fission machinery as demonstrated by the higher oligomerization and increased translocation of Drp1 to mitochondria. Moreover, we observed alterations in the processing of OPA1 in cells bearing the mutation in huntingtin gene. Huntington's disease cells displayed enhanced susceptibility to cell death and faster release of cytochrome c in response to intrinsic apoptotic stimuli. We were able to correct the morphological phenotype by overexpressing pro-fusion proteins and inhibiting the fission machinery. However the protection from cell death was achieved only when the calcineurin-Drp1 axis was inhibited or OPA1 was overexpressed, but not upon overexpression of MFN1. Moreover, overexpression of OPA1 and a dominant negative form of Drp1 could restore normal *cristae* structure and protect from *cristae* remodelling during apoptosis. These data suggest that regulation of mitochondrial morphology *per se* is not sufficient to protect from cell death, but it has to correlate with preservation of mitochondrial ultrastructure. While the role of OPA1 in the regulation of *cristae* structure has been well characterized, the precise mechanism by which inhibition of Drp1 affects mitochondrial ultrastructure remains unclear. Recent reports show that Drp1 ablation causes changes in the cleavage of the inner membrane Opa1 (Mopert et al., 2009) raising the possibility that the pro-fission dynamin-related protein could indirectly

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influence *cristae* morphology acting of OPA1 regulation and activity. In Huntington's disease the picture is even more complicated because of the presence of mitochondrial metabolic defects, being OPA1 processing dependent on ATP levels (Baricault et al., 2007). The data collected so far are not sufficient to understand which is the primary cause of the observed mitochondrial defects but our results show that regulation of morphology and ultrastructure can dominantly affect cell death. Moreover, recent reports show that correction of mitochondrial shape can restore ATP production in cells bearing mutated huntingtin (Wang et al., 2008), confirming the intimate link between mitochondrial shape and bioenergetics and further supporting the importance of mitochondrial dynamics in the disease. Although a single gene mutation causes Huntington's disease, the outcome is highly complex and our work characterized a novel pathological mechanism and identified molecular players that could serve as potential therapeutic targets.

6 Reference List

 (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell. *72*, 971-983.

Alavi,M.V., Bette,S., Schimpf,S., Schuettauf,F., Schraermeyer,U., Wehrl,H.F., Ruttiger,L., Beck,S.C., Tonagel,F., Pichler,B.J., Knipper,M., Peters,T., Laufs,J., and Wissinger,B. (2007). A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy. Brain. *130*, 1029-1042.

Alexander,C., Votruba,M., Pesch,U.E., Thiselton,D.L., Mayer,S., Moore,A., Rodriguez,M., Kellner,U., Leo-Kottler,B., Auburger,G., Bhattacharya,S.S., and Wissinger,B. (2000). OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat. Genet. *26*, 211-215.

Alirol,E., James,D., Huber,D., Marchetto,A., Vergani,L., Martinou,J.C., and Scorrano,L. (2006). The mitochondrial fission protein hFis1 requires the endoplasmic reticulum gateway to induce apoptosis. Mol. Biol. Cell. *17*, 4593-4605.

Allen,J.F., Puthiyaveetil,S., Strom,J., and Allen,C.A. (2005). Energy transduction anchors genes in organelles. Bioessays. *27*, 426-435.

Almeida,S., Domingues,A., Rodrigues,L., Oliveira,C.R., and Rego,A.C. (2004). FK506 prevents mitochondrial-dependent apoptotic cell death induced by 3-nitropropionic acid in rat primary cortical cultures. Neurobiol. Dis. *17*, 435-444.

Almeida,S., Sarmento-Ribeiro,A.B., Januario,C., Rego,A.C., and Oliveira,C.R. (2008). Evidence of apoptosis and mitochondrial abnormalities in peripheral blood cells of Huntington's disease patients. Biochem. Biophys. Res. Commun. *374*, 599-603.

Amati-Bonneau,P., Pasquier,L., Lainey,E., Ferre,M., Odent,S., Malthiery,Y., Bonneau,D., and Reynier,P. (2005a). Sporadic optic atrophy due to synonymous codon change altering mRNA splicing of OPA1. Clin. Genet. *67*, 102-103.

Amati-Bonneau,P., Pasquier,L., Lainey,E., Ferre,M., Odent,S., Malthiery,Y., Bonneau,D., and Reynier,P. (2005b). Sporadic optic atrophy due to synonymous codon change altering mRNA splicing of OPA1. Clin. Genet. *67*, 102-103.

Amchenkova,A.A., Bakeeva,L.E., Chentsov,Y.S., Skulachev,V.P., and Zorov,D.B. (1988). Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes. J. Cell Biol. *107*, 481-495.

Amutha,B., Gordon,D.M., Gu,Y., and Pain,D. (2004). A novel role of Mgm1p, a dynamin-related GTPase, in ATP synthase assembly and cristae formation/maintenance. Biochem. J. *381*, 19-23.

Anderson,L. (1981). Identification of mitochondrial proteins and some of their precursors in two-dimensional electrophoretic maps of human cells. Proc. Natl. Acad. Sci. U. S. A. *78*, 2407-2411.

Andreassen,O.A., Ferrante,R.J., Huang,H.M., Dedeoglu,A., Park,L., Ferrante,K.L., Kwon,J., Borchelt,D.R., Ross,C.A., Gibson,G.E., and Beal,M.F. (2001). Dichloroacetate exerts therapeutic effects in transgenic mouse models of Huntington's disease. Ann. Neurol. *50*, 112-117.

Andresen,J.M., Gayan,J., Djousse,L., Roberts,S., Brocklebank,D., Cherny,S.S., US-Venezuela Collaborative Research Group, HD MAPS Collaborative Research Group, Cardon,L.R., Gusella,J.F., MacDonald,M.E., Myers,R.H., Housman,D.E., and Wexler,N.S. (2007). The relationship between CAG repeat length and age of onset differs for Huntington's disease patients with juvenile onset or adult onset. Ann. Hum. Genet. *71*, 295-301.

Andrews,R.M., Griffiths,P.G., Johnson,M.A., and Turnbull,D.M. (1999). Histochemical localisation of mitochondrial enzyme activity in human optic nerve and retina. Br. J. Ophthalmol. *83*, 231-235.

Antonini,A., Leenders,K.L., Spiegel,R., Meier,D., Vontobel,P., Weigell-Weber,M., Sanchez-Pernaute,R., de Yebenez,J.G., Boesiger,P., Weindl,A., and Maguire,R.P. (1996). Striatal glucose metabolism and dopamine D2 receptor binding in asymptomatic gene carriers and patients with Huntington's disease. Brain. *119 (Pt 6)*, 2085-2095.

Arnoult,D., Grodet,A., Lee,Y.J., Estaquier,J., and Blackstone,C. (2005). Release of OPA1 during apoptosis participates in the rapid and complete release of cytochrome c and subsequent mitochondrial fragmentation. J. Biol. Chem. *280*, 35742-35750.

Arrasate,M., Mitra,S., Schweitzer,E.S., Segal,M.R., and Finkbeiner,S. (2004). Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature. *431*, 805-810.

Arzberger,T., Krampfl,K., Leimgruber,S., and Weindl,A. (1997). Changes of NMDA receptor subunit (NR1, NR2B) and glutamate transporter (GLT1) mRNA expression in Huntington's disease--an in situ hybridization study. J. Neuropathol. Exp. Neurol. *56*, 440-454.

Attardi,G. and Schatz,G. (1988). Biogenesis of mitochondria. Annu. Rev. Cell. Biol. *4*, 289-333.

Bach,D., Pich,S., Soriano,F.X., Vega,N., Baumgartner,B., Oriola,J., Daugaard,J.R., Lloberas,J., Camps,M., Zierath,J.R., Rabasa-Lhoret,R., Wallberg-Henriksson,H., Laville,M., Palacin,M., Vidal,H., Rivera,F., Brand,M., and Zorzano,A. (2003). Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. J. Biol. Chem. *278*, 17190-17197.

Bae,B.I., Xu,H., Igarashi,S., Fujimuro,M., Agrawal,N., Taya,Y., Hayward,S.D., Moran,T.H., Montell,C., Ross,C.A., Snyder,S.H., and Sawa,A. (2005). p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. Neuron. *47*, 29-41.

Baricault,L., Segui,B., Guegand,L., Olichon,A., Valette,A., Larminat,F., and Lenaers,G. (2007). OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals. Exp. Cell. Res. *313*, 3800-3808.

Barsoum,M.J., Yuan,H., Gerencser,A.A., Liot,G., Kushnareva,Y., Graber,S., Kovacs,I., Lee,W.D., Waggoner,J., Cui,J., White,A.D., Bossy,B., Martinou,J.C., Youle,R.J., Lipton,S.A., Ellisman,M.H., Perkins,G.A., and Bossy-Wetzel,E. (2006). Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. EMBO. J. *25*, 3900-3911.

Behrens,P.F., Franz,P., Woodman,B., Lindenberg,K.S., and Landwehrmeyer,G.B. (2002). Impaired glutamate transport and glutamate-glutamine cycling: downstream effects of the Huntington mutation. Brain. *125*, 1908-1922.

Benard,G., Bellance,N., James,D., Parrone,P., Fernandez,H., Letellier,T., and Rossignol,R. (2007). Mitochondrial bioenergetics and structural network organization. J. Cell. Sci. *120*, 838-848.

Bence,N.F., Sampat,R.M., and Kopito,R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science. *292*, 1552-1555.

Bennett,E.J., Shaler,T.A., Woodman,B., Ryu,K.Y., Zaitseva,T.S., Becker,C.H., Bates,G.P., Schulman,H., and Kopito,R.R. (2007). Global changes to the ubiquitin system in Huntington's disease. Nature. *448*, 704-708.

Berger,K.H. and Yaffe,M.P. (1996). Mitochondrial distribution and inheritance. Experientia. *52*, 1111-1116.

Berger,Z., Ravikumar,B., Menzies,F.M., Oroz,L.G., Underwood,B.R., Pangalos,M.N., Schmitt,I., Wullner,U., Evert,B.O., O'Kane,C.J., and Rubinsztein,D.C. (2006). Rapamycin alleviates toxicity of different aggregate-prone proteins. Hum. Mol. Genet. *15*, 433-442.

Berliocchi,L., Bano,D., and Nicotera,P. (2005). Ca2+ signals and death programmes in neurons. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *360*, 2255-2258.

Bernardi,P. and Azzone,G.F. (1981). Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. J. Biol. Chem. *256*, 7187-7192.

Bernardi,P., Petronilli,V., Di Lisa,F., and Forte,M. (2001). A mitochondrial perspective on cell death. Trends. Biochem. Sci. *26*, 112-117.

Blackinton,J., Lakshminarasimhan,M., Thomas,K.J., Ahmad,R., Greggio,E., Raza,A.S., Cookson,M.R., and Wilson,M.A. (2009). Formation of a stabilized cysteine sulfinic acid is critical for the mitochondrial function of the parkinsonism protein DJ-1. J. Biol. Chem. *284*, 6476-6485.

Bonelli,R.M., Heuberger,C., and Reisecker,F. (2003). Minocycline for Huntington's disease: an open label study. Neurology. *60*, 883-884.

Braak,H. and Braak,E. (1992). Allocortical involvement in Huntington's disease. Neuropathol. Appl. Neurobiol. *18*, 539-547.

Braschi,E., Zunino,R., and McBride,H.M. (2009). MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. EMBO. Rep. *10*, 748-754.

Breckenridge,D.G., Stojanovic,M., Marcellus,R.C., and Shore,G.C. (2003). Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J. Cell Biol. *160*, 1115-1127.

Brennan,W.A., Jr., Bird,E.D., and Aprille,J.R. (1985). Regional mitochondrial respiratory activity in Huntington's disease brain. J. Neurochem. *44*, 1948-1950.

Bristow,E.A., Griffiths,P.G., Andrews,R.M., Johnson,M.A., and Turnbull,D.M. (2002). The distribution of mitochondrial activity in relation to optic nerve structure. Arch. Ophthalmol. *120*, 791-796.

Brooks,C., Wei,Q., Feng,L., Dong,G., Tao,Y., Mei,L., Xie,Z.J., and Dong,Z. (2007). Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc. Natl. Acad. Sci. U. S. A. *104*, 11649-11654.

Browne,S.E. and Beal,M.F. (2002). Toxin-induced mitochondrial dysfunction. Int. Rev. Neurobiol. *53*, 243-279.

Browne,S.E. and Beal,M.F. (2006). Oxidative damage in Huntington's disease pathogenesis. Antioxid. Redox. Signal. *8*, 2061-2073.

Browne,S.E., Bowling,A.C., MacGarvey,U., Baik,M.J., Berger,S.C., Muqit,M.M., Bird,E.D., and Beal,M.F. (1997a). Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann. Neurol. *41*, 646-653.

Browne,S.E., Bowling,A.C., MacGarvey,U., Baik,M.J., Berger,S.C., Muqit,M.M., Bird,E.D., and Beal,M.F. (1997b). Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann. Neurol. *41*, 646-653.

Browne,S.E., Ferrante,R.J., and Beal,M.F. (1999). Oxidative stress in Huntington's disease. Brain. Pathol. *9*, 147-163.

Calvo,S., Jain,M., Xie,X., Sheth,S.A., Chang,B., Goldberger,O.A., Spinazzola,A., Zeviani,M., Carr,S.A., and Mootha,V.K. (2006). Systematic identification of human mitochondrial disease genes through integrative genomics. Nat. Genet. *38*, 576-582.

Campello,S., Lacalle,R.A., Bettella,M., Manes,S., Scorrano,L., and Viola,A. (2006). Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. J. Exp. Med. *203*, 2879-2886.

Carelli,V., Ross-Cisneros,F.N., and Sadun,A.A. (2004). Mitochondrial dysfunction as a cause of optic neuropathies. Prog. Retin. Eye. Res. *23*, 53-89.

Catlett,N.L. and Weisman,L.S. (2000). Divide and multiply: organelle partitioning in yeast. Curr. Opin. Cell. Biol. *12*, 509-516.

Cepeda,C., Hurst,R.S., Calvert,C.R., Hernandez-Echeagaray,E., Nguyen,O.K., Jocoy,E., Christian,L.J., Ariano,M.A., and Levine,M.S. (2003). Transient and

progressive electrophysiological alterations in the corticostriatal pathway in a mouse model of Huntington's disease. J. Neurosci. *23*, 961-969.

Cereghetti,G.M., Stangherlin,A., Martins,d.B., Chang,C.R., Blackstone,C., Bernardi,P., and Scorrano,L. (2008). Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. U. S. A *105*, 15803-15808.

Cha,J.H., Kosinski,C.M., Kerner,J.A., Alsdorf,S.A., Mangiarini,L., Davies,S.W., Penney,J.B., Bates,G.P., and Young,A.B. (1998). Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human huntington disease gene. Proc. Natl. Acad. Sci. U. S. A. *95*, 6480-6485.

Chan,D.C. (2006). Mitochondria: dynamic organelles in disease, aging, and development. Cell. *125*, 1241-1252.

Chang,C.R. and Blackstone,C. (2007). Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J. Biol. Chem. *282*, 21583-21587.

Chen,H., Chomyn,A., and Chan,D.C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J. Biol. Chem. *280*, 26185-26192.

Chen,H., Detmer,S.A., Ewald,A.J., Griffin,E.E., Fraser,S.E., and Chan,D.C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J. Cell Biol. *160*, 189-200.

Chen,H., McCaffery,J.M., and Chan,D.C. (2007). Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell. *130*, 548-562.

Chen,K.H., Guo,X., Ma,D., Guo,Y., Li,Q., Yang,D., Li,P., Qiu,X., Wen,S., Xiao,R.P., and Tang,J. (2004). Dysregulation of HSG triggers vascular proliferative disorders. Nat. Cell Biol. *6*, 872-883.

Cheng,E.H., Wei,M.C., Weiler,S., Flavell,R.A., Mak,T.W., Lindsten,T., and Korsmeyer,S.J. (2001). Bcl-2, bcl-x(l) sequester bh3 domain-only molecules preventing bax- and bak-mediated mitochondrial apoptosis. Mol. Cell *8*, 705-711.

Cherra,S.J., III, Dagda,R.K., Tandon,A., and Chu,C.T. (2009). Mitochondrial autophagy as a compensatory response to PINK1 deficiency. Autophagy. *5*, 1213-1214.

Cho,D.H., Nakamura,T., Fang,J., Cieplak,P., Godzik,A., Gu,Z., and Lipton,S.A. (2009). S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science. *324*, 102-105.

Choi,S.Y., Huang,P., Jenkins,G.M., Chan,D.C., Schiller,J., and Frohman,M.A. (2006). A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. Nat. Cell. Biol. *8*, 1255-1262.

Choo,Y.S., Johnson,G.V., MacDonald,M., Detloff,P.J., and Lesort,M. (2004a). Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. Hum. Mol. Genet. *13*, 1407-1420.

Choo,Y.S., Johnson,G.V., MacDonald,M., Detloff,P.J., and Lesort,M. (2004b). Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. Hum. Mol. Genet. *13*, 1407-1420.

Ciammola,A., Sassone,J., Alberti,L., Meola,G., Mancinelli,E., Russo,M.A., Squitieri,F., and Silani,V. (2006). Increased apoptosis, Huntingtin inclusions and altered differentiation in muscle cell cultures from Huntington's disease subjects. Cell. Death. Differ. *13*, 2068-2078.

Cipolat,S., de Brito,O.M., Dal Zilio,B., and Scorrano,L. (2004). OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc. Natl. Acad. Sci. U. S. A *101*, 15927-15932.

Cipolat,S., Rudka,T., Hartmann,D., Costa,V., Serneels,L., Craessaerts,K., Metzger,K., Frezza,C., Annaert,W., D'Adamio,L., Derks,C., Dejaegere,T., Pellegrini,L., D'Hooge,R., Scorrano,L., and De Strooper,B. (2006). Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell *126*, 163-175.

Clark,I.E., Dodson,M.W., Jiang,C., Cao,J.H., Huh,J.R., Seol,J.H., Yoo,S.J., Hay,B.A., and Guo,M. (2006). Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature. *441*, 1162-1166.

Cohen,M.M., Leboucher,G.P., Livnat-Levanon,N., Glickman,M.H., and Weissman,A.M. (2008). Ubiquitin-proteasome-dependent degradation of a mitofusin, a critical regulator of mitochondrial fusion. Mol. Biol. Cell. *19*, 2457-2464.

Collins,T.J., Berridge,M.J., Lipp,P., and Bootman,M.D. (2002). Mitochondria are morphologically and functionally heterogeneous within cells. EMBO J. *21*, 1616-1627.

Corral-Debrinski,M., Horton,T., Lott,M.T., Shoffner,J.M., Beal,M.F., and Wallace,D.C. (1992). Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nat. Genet. *2*, 324-329.

Cowan,K.J., Diamond,M.I., and Welch,W.J. (2003). Polyglutamine protein aggregation and toxicity are linked to the cellular stress response. Hum. Mol. Genet. *12*, 1377-1391.

Cribbs,J.T. and Strack,S. (2007). Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO. Rep. *8*, 939-944.

D'Herde,K., De Prest,B., Mussche,S., Schotte,P., Beyaert,R., Coster,R.V., and Roels,F. (2001). Ultrastructural localization of cytochrome c in apoptosis demonstrates mitochondrial heterogeneity. Cell Death. Differ. *7*, 331-337.

Dagda,R.K. and Chu,C.T. (2009). Mitochondrial quality control: insights on how Parkinson's disease related genes PINK1, parkin, and Omi/HtrA2 interact to maintain mitochondrial homeostasis. J. Bioenerg. Biomembr.

Danial,N.N. and Korsmeyer,S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.

Davies,V.J., Hollins,A.J., Piechota,M.J., Yip,W., Davies,J.R., White,K.E., Nicols,P.P., Boulton,M.E., and Votruba,M. (2007). Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. Hum. Mol. Genet. *16*, 1307-1318.

de Brito,O.M. and Scorrano,L. (2008). Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature. *456*, 605-610.

De Rooij,K.E., Dorsman,J.C., Smoor,M.A., Den Dunnen,J.T., and Van Ommen,G.J. (1996). Subcellular localization of the Huntington's disease gene product in cell lines by immunofluorescence and biochemical subcellular fractionation. Hum. Mol. Genet. *5*, 1093-1099.

De Vos,K., Severin,F., Van Herreweghe,F., Vancompernolle,K., Goossens,V., Hyman,A., and Grooten,J. (2000). Tumor necrosis factor induces hyperphosphorylation of kinesin light chain and inhibits kinesin-mediated transport of mitochondria. J. Cell. Biol. *149*, 1207-1214.

De Vos,K.J., Allan,V.J., Grierson,A.J., and Sheetz,M.P. (2005). Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. Curr. Biol. *15*, 678-683.

Delettre,C., Griffoin,J.M., Kaplan,J., Dollfus,H., Lorenz,B., Faivre,L., Lenaers,G., Belenguer,P., and Hamel,C.P. (2001). Mutation spectrum and splicing variants in the OPA1 gene. Hum. Genet. *109*, 584-591.

Delettre,C., Lenaers,G., Griffoin,J.M., Gigarel,N., Lorenzo,C., Belenguer,P., Pelloquin,L., Grosgeorge,J., Turc-Carel,C., Perret,E., Astarie-Dequeker,C., Lasquellec,L., Arnaud,B., Ducommun,B., Kaplan,J., and Hamel,C.P. (2000). Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat. Genet. *26*, 207-210.

Delettre,C., Lenaers,G., Pelloquin,L., Belenguer,P., and Hamel,C.P. (2002). OPA1 (Kjer type) dominant optic atrophy: a novel mitochondrial disease. Mol. Genet. Metab. *75*, 97-107.

Deng,H., Dodson,M.W., Huang,H., and Guo,M. (2008). The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *105*, 14503-14508.

Di Bacco,A. and Gill,G. (2006). SUMO-specific proteases and the cell cycle. An essential role for SENP5 in cell proliferation. Cell. Cycle. *5*, 2310-2313.

DiFiglia,M., Sapp,E., Chase,K., Schwarz,C., Meloni,A., Young,C., Martin,E., Vonsattel,J.P., Carraway,R., Reeves,S.A., and . (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. Neuron. *14*, 1075-1081.

DiFiglia,M., Sapp,E., Chase,K.O., Davies,S.W., Bates,G.P., Vonsattel,J.P., and Aronin,N. (1997a). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. *277*, 1990-1993.

DiFiglia,M., Sapp,E., Chase,K.O., Davies,S.W., Bates,G.P., Vonsattel,J.P., and Aronin,N. (1997b). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. *277*, 1990-1993.

Dimmer,K.S., Fritz,S., Fuchs,F., Messerschmitt,M., Weinbach,N., Neupert,W., and Westermann,B. (2002). Genetic basis of mitochondrial function and morphology in Saccharomyces cerevisiae. Mol. Biol. Cell *13*, 847-853.

Djousse,L., Knowlton,B., Cupples,L.A., Marder,K., Shoulson,I., and Myers,R.H. (2002a). Weight loss in early stage of Huntington's disease. Neurology. *59*, 1325-1330.

Djousse,L., Knowlton,B., Cupples,L.A., Marder,K., Shoulson,I., and Myers,R.H. (2002b). Weight loss in early stage of Huntington's disease. Neurology. *59*, 1325-1330.

Dragatsis,I., Levine,M.S., and Zeitlin,S. (2000). Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat. Genet. *26*, 300-306.

Dragunow,M., Faull,R.L., Lawlor,P., Beilharz,E.J., Singleton,K., Walker,E.B., and Mee,E. (1995a). In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. Neuroreport. *6*, 1053-1057.

Dragunow,M., Faull,R.L., Lawlor,P., Beilharz,E.J., Singleton,K., Walker,E.B., and Mee,E. (1995b). In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. Neuroreport. *6*, 1053-1057.

Dudkina,N.V., Heinemeyer,J., Keegstra,W., Boekema,E.J., and Braun,H.P. (2005). Structure of dimeric ATP synthase from mitochondria: an angular association of monomers induces the strong curvature of the inner membrane. FEBS. Lett. *579*, 5769-5772.

Duvezin-Caubet,S., Jagasia,R., Wagener,J., Hofmann,S., Trifunovic,A., Hansson,A., Chomyn,A., Bauer,M.F., Attardi,G., Larsson,N.G., Neupert,W., and Reichert,A.S. (2006). Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J. Biol. Chem. *281*, 37972-37979.

Duvezin-Caubet,S., Koppen,M., Wagener,J., Zick,M., Israel,L., Bernacchia,A., Jagasia,R., Rugarli,E.I., Imhof,A., Neupert,W., Langer,T., and Reichert,A.S. (2007). OPA1 processing reconstituted in yeast depends on the subunit composition of the m-AAA protease in mitochondria. Mol. Biol. Cell. *18*, 3582-3590.

Duyao,M., Ambrose,C., Myers,R., Novelletto,A., Persichetti,F., Frontali,M., Folstein,S., Ross,C., Franz,M., Abbott,M., and . (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat. Genet. *4*, 387-392.

Escobar-Henriques,M., Westermann,B., and Langer,T. (2006). Regulation of mitochondrial fusion by the F-box protein Mdm30 involves proteasome-independent turnover of Fzo1. J. Cell. Biol. *173*, 645-650.

Esser,K., Tursun,B., Ingenhoven,M., Michaelis,G., and Pratje,E. (2002). A novel two-step mechanism for removal of a mitochondrial signal sequence involves the mAAA complex and the putative rhomboid protease Pcp1. J. Mol. Biol. *323*, 835-843.

Eura,Y., Ishihara,N., Oka,T., and Mihara,K. (2006). Identification of a novel protein that regulates mitochondrial fusion by modulating mitofusin (Mfn) protein function. J. Cell. Sci. *119*, 4913-4925.

Exner,N., Treske,B., Paquet,D., Holmstrom,K., Schiesling,C., Gispert,S., Carballo-Carbajal,I., Berg,D., Hoepken,H.H., Gasser,T., Kruger,R., Winklhofer,K.F., Vogel,F., Reichert,A.S., Auburger,G., Kahle,P.J., Schmid,B., and Haass,C. (2007). Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. J. Neurosci. *27*, 12413-12418.

Faber,P.W., Alter,J.R., MacDonald,M.E., and Hart,A.C. (1999). Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron. Proc. Natl. Acad. Sci. U. S. A. *96*, 179-184.

Fan,J., Cowan,C.M., Zhang,L.Y., Hayden,M.R., and Raymond,L.A. (2009). Interaction of postsynaptic density protein-95 with NMDA receptors influences excitotoxicity in the yeast artificial chromosome mouse model of Huntington's disease. J. Neurosci. *29*, 10928-10938.

Fehrenbacher,K.L., Yang,H.C., Gay,A.C., Huckaba,T.M., and Pon,L.A. (2004). Live cell imaging of mitochondrial movement along actin cables in budding yeast. Curr. Biol. *14*, 1996-2004.

Fernandes,H.B., Baimbridge,K.G., Church,J., Hayden,M.R., and Raymond,L.A. (2007). Mitochondrial sensitivity and altered calcium handling underlie enhanced NMDA-induced apoptosis in YAC128 model of Huntington's disease. J. Neurosci. *27*, 13614-13623.

Fernandez-Silva,P., Enriquez,J.A., and Montoya,J. (2003). Replication and transcription of mammalian mitochondrial DNA. Exp. Physiol. *88*, 41-56.

Ferrante,R.J., Andreassen,O.A., Dedeoglu,A., Ferrante,K.L., Jenkins,B.G., Hersch,S.M., and Beal,M.F. (2002). Therapeutic effects of coenzyme Q10 and remacemide in transgenic mouse models of Huntington's disease. J. Neurosci. *22*, 1592-1599.

Ferrante,R.J., Andreassen,O.A., Jenkins,B.G., Dedeoglu,A., Kuemmerle,S., Kubilus,J.K., Kaddurah-Daouk,R., Hersch,S.M., and Beal,M.F. (2000). Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. J. Neurosci. *20*, 4389-4397.

Ferrante,R.J., Kowall,N.W., and Richardson,E.P., Jr. (1991). Proliferative and degenerative changes in striatal spiny neurons in Huntington's disease: a combined study using the section-Golgi method and calbindin D28k immunocytochemistry. J. Neurosci. *11*, 3877-3887.

Ferre,M., Amati-Bonneau,P., Tourmen,Y., Malthiery,Y., and Reynier,P. (2005a). eOPA1: an online database for OPA1 mutations. Hum. Mutat. *25*, 423-428.

Ferre,M., Amati-Bonneau,P., Tourmen,Y., Malthiery,Y., and Reynier,P. (2005b). eOPA1: an online database for OPA1 mutations. Hum. Mutat. *25*, 423-428.

Frank,S., Gaume,B., Bergmann-Leitner,E.S., Leitner,W.W., Robert,E.G., Catez,F., Smith,C.L., and Youle,R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell *1*, 515-525.

Frederick,R.L., McCaffery,J.M., Cunningham,K.W., Okamoto,K., and Shaw,J.M. (2004). Yeast Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway. J. Cell. Biol. *167*, 87-98.

Frey,T.G. and Mannella,C.A. (2000). The internal structure of mitochondria. Trends. Biochem. Sci. *25*, 319-324.

Frezza,C., Cipolat,S., Martins,d.B., Micaroni,M., Beznoussenko,G.V., Rudka,T., Bartoli,D., Polishuck,R.S., Danial,N.N., De Strooper,B., and Scorrano,L. (2006). OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell *126*, 177-189.

Frieden,M., James,D., Castelbou,C., Danckaert,A., Martinou,J.C., and Demaurex,N. (2004). Ca(2+) homeostasis during mitochondrial fragmentation and perinuclear clustering induced by hFis1. J. Biol. Chem. *279*, 22704-22714.

Gafni,J. and Ellerby,L.M. (2002). Calpain activation in Huntington's disease. J. Neurosci. *22*, 4842-4849.

Gallop,J.L., Jao,C.C., Kent,H.M., Butler,P.J., Evans,P.R., Langen,R., and McMahon,H.T. (2006). Mechanism of endophilin N-BAR domain-mediated membrane curvature. EMBO. J. *25*, 2898-2910.

Gallop,J.L. and McMahon,H.T. (2005). BAR domains and membrane curvature: bringing your curves to the BAR. Biochem. Soc. Symp. 223-231.

Gandre-Babbe,S. and van der Bliek,A.M. (2008). The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol. Biol. Cell. *19*, 2402-2412.

Gauthier,L.R., Charrin,B.C., Borrell-Pages,M., Dompierre,J.P., Rangone,H., Cordelieres,F.P., De Mey,J., MacDonald,M.E., Lessmann,V., Humbert,S., and Saudou,F. (2004). Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell. *118*, 127-138.

Gavrieli,Y., Sherman,Y., and Ben Sasson,S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell. Biol. *119*, 493-501.

Germain,M., Mathai,J.P., McBride,H.M., and Shore,G.C. (2005). Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. EMBO J. *24*, 1546-1556.

Gervais,F.G., Singaraja,R., Xanthoudakis,S., Gutekunst,C.A., Leavitt,B.R., Metzler,M., Hackam,A.S., Tam,J., Vaillancourt,J.P., Houtzager,V., Rasper,D.M., Roy,S., Hayden,M.R., and Nicholson,D.W. (2002). Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. Nat. Cell. Biol. *4*, 95-105.

Gieffers,C., Korioth,F., Heimann,P., Ungermann,C., and Frey,J. (1997). Mitofilin is a transmembrane protein of the inner mitochondrial membrane expressed as two isoforms. Exp. Cell Res. *232*, 395-399.

Gines,S., Seong,I.S., Fossale,E., Ivanova,E., Trettel,F., Gusella,J.F., Wheeler,V.C., Persichetti,F., and MacDonald,M.E. (2003). Specific progressive cAMP reduction implicates energy deficit in presymptomatic Huntington's disease knock-in mice. Hum. Mol. Genet. *12*, 497-508.

Gizatullina,Z.Z., Lindenberg,K.S., Harjes,P., Chen,Y., Kosinski,C.M., Landwehrmeyer,B.G., Ludolph,A.C., Striggow,F., Zierz,S., and Gellerich,F.N. (2006). Low stability of Huntington muscle mitochondria against Ca2+ in R6/2 mice. Ann. Neurol. *59*, 407-411.

Glater,E.E., Megeath,L.J., Stowers,R.S., and Schwarz,T.L. (2006). Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent. J. Cell. Biol. *173*, 545-557.

Goebel,H.H., Heipertz,R., Scholz,W., Iqbal,K., and Tellez-Nagel,I. (1978). Juvenile Huntington chorea: clinical, ultrastructural, and biochemical studies. Neurology. *28*, 23-31.

Graham,R.K., Deng,Y., Slow,E.J., Haigh,B., Bissada,N., Lu,G., Pearson,J., Shehadeh,J., Bertram,L., Murphy,Z., Warby,S.C., Doty,C.N., Roy,S., Wellington,C.L., Leavitt,B.R., Raymond,L.A., Nicholson,D.W., and Hayden,M.R. (2006). Cleavage at the caspase-6 site is required for neuronal dysfunction and degeneration due to mutant huntingtin. Cell. *125*, 1179-1191.

Griparic,L., van der Wel,N.N., Orozco,I.J., Peters,P.J., and van der Bliek,A.M. (2004). Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. J. Biol. Chem. *279*, 18792-18798.

Grunewald,A., Gegg,M.E., Taanman,J.W., King,R.H., Kock,N., Klein,C., and Schapira,A.H. (2009). Differential effects of PINK1 nonsense and missense mutations on mitochondrial function and morphology. Exp. Neurol. *219*, 266-273.

Grunewald,T. and Beal,M.F. (1999). Bioenergetics in Huntington's disease. Ann. N. Y. Acad. Sci. *893*, 203-213.

Guillery,O., Malka,F., Landes,T., Guillou,E., Blackstone,C., Lombes,A., Belenguer,P., Arnoult,D., and Rojo,M. (2008). Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. Biol. Cell. *100*, 315-325.

Gunawardena,S., Her,L.S., Brusch,R.G., Laymon,R.A., Niesman,I.R., Gordesky-Gold,B., Sintasath,L., Bonini,N.M., and Goldstein,L.S. (2003). Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila. Neuron. *40*, 25-40.

Guo,X., Macleod,G.T., Wellington,A., Hu,F., Panchumarthi,S., Schoenfield,M., Marin,L., Charlton,M.P., Atwood,H.L., and Zinsmaier,K.E. (2005). The GTPase dMiro is required for axonal transport of mitochondria to Drosophila synapses. Neuron. *47*, 379-393.

Gusella,J.F. and Macdonald,M.E. (2009). Huntington's disease: the case for genetic modifiers. Genome. Med. *1*, 80.

Gutekunst,C.A., Levey,A.I., Heilman,C.J., Whaley,W.L., Yi,H., Nash,N.R., Rees,H.D., Madden,J.J., and Hersch,S.M. (1995). Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. Proc. Natl. Acad. Sci. U. S. A. *92*, 8710-8714.

Gutekunst,C.A., Li,S.H., Yi,H., Ferrante,R.J., Li,X.J., and Hersch,S.M. (1998). The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. J. Neurosci. *18*, 7674-7686.

Gutekunst,C.A., Li,S.H., Yi,H., Mulroy,J.S., Kuemmerle,S., Jones,R., Rye,D., Ferrante,R.J., Hersch,S.M., and Li,X.J. (1999a). Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J. Neurosci. *19*, 2522-2534.

Gutekunst,C.A., Li,S.H., Yi,H., Mulroy,J.S., Kuemmerle,S., Jones,R., Rye,D., Ferrante,R.J., Hersch,S.M., and Li,X.J. (1999b). Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J. Neurosci. *19*, 2522-2534.

Hackam,A.S., Singaraja,R., Wellington,C.L., Metzler,M., McCutcheon,K., Zhang,T., Kalchman,M., and Hayden,M.R. (1998). The influence of huntingtin protein size on nuclear localization and cellular toxicity. J. Cell. Biol. *141*, 1097-1105.

Hackenbrock,C.R. (1966). Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J. Cell Biol. *30*, 269-297.

Hacker,G. (2000). The morphology of apoptosis. Cell. Tissue. Res. *301*, 5-17.

Hales,K.G. and Fuller,M.T. (1997). Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. Cell *90*, 121-129.

Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. Cell. *100*, 57-70.

Hansson,O., Guatteo,E., Mercuri,N.B., Bernardi,G., Li,X.J., Castilho,R.F., and Brundin,P. (2001). Resistance to NMDA toxicity correlates with appearance of nuclear inclusions, behavioural deficits and changes in calcium homeostasis in mice transgenic for exon 1 of the huntington gene. Eur. J. Neurosci. *14*, 1492-1504.

Hantraye,P., Riche,D., Maziere,M., and Isacson,O. (1990). A primate model of Huntington's disease: behavioral and anatomical studies of unilateral excitotoxic lesions of the caudate-putamen in the baboon. Exp. Neurol. *108*, 91-104.

Harder,Z., Zunino,R., and McBride,H. (2004). Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr. Biol. *14*, 340-345.

Harper,P.S. (1991). The case against. Nature. *353*, 9.

Hayden,M.R., Berkowicz,A.L., Beighton,P.H., and Yiptong,C. (1981). Huntington's chorea on the island of Mauritius. S. Afr. Med. J. *60*, 1001-1002.

Hebb,C. (1970). CNS at the cellular level: identity of transmitter agents. Annu. Rev. Physiol. *32*, 165-192.

Hedreen,J.C. and Folstein,S.E. (1995). Early loss of neostriatal striosome neurons in Huntington's disease. J. Neuropathol. Exp. Neurol. *54*, 105-120.

Henchcliffe,C., Shungu,D.C., Mao,X., Huang,C., Nirenberg,M.J., Jenkins,B.G., and Beal,M.F. (2008). Multinuclear magnetic resonance spectroscopy for in vivo assessment of mitochondrial dysfunction in Parkinson's disease. Ann. N. Y. Acad. Sci. *1147*, 206-220.

Hengartner,M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Herlan,M., Vogel,F., Bornhovd,C., Neupert,W., and Reichert,A.S. (2003). Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. J. Biol. Chem. *278*, 27781-27788.

Hermann,G.J. and Shaw,J.M. (1998). Mitochondrial dynamics in yeast. Annu. Rev. Cell. Dev. Biol. *14*, 265-303.

Hermann,G.J., Thatcher,J.W., Mills,J.P., Hales,K.G., Fuller,M.T., Nunnari,J., and Shaw,J.M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J. Cell Biol. *143*, 359-373.

Hickey,M.A. and Chesselet,M.F. (2003). Apoptosis in Huntington's disease. Prog. Neuropsychopharmacol. Biol. Psychiatry. *27*, 255-265.

Hinshaw,J.E. (1999). Dynamin spirals. Curr. Opin. Struct. Biol. *9*, 260-267.

Ho,L.W., Carmichael,J., Swartz,J., Wyttenbach,A., Rankin,J., and Rubinsztein,D.C. (2001). The molecular biology of Huntington's disease. Psychol. Med. *31*, 3-14.

Hodgson,J.G., Agopyan,N., Gutekunst,C.A., Leavitt,B.R., LePiane,F., Singaraja,R., Smith,D.J., Bissada,N., McCutcheon,K., Nasir,J., Jamot,L., Li,X.J., Stevens,M.E., Rosemond,E., Roder,J.C., Phillips,A.G., Rubin,E.M., Hersch,S.M., and Hayden,M.R. (1999a). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron. *23*, 181-192.

Hodgson,J.G., Agopyan,N., Gutekunst,C.A., Leavitt,B.R., LePiane,F., Singaraja,R., Smith,D.J., Bissada,N., McCutcheon,K., Nasir,J., Jamot,L., Li,X.J., Stevens,M.E., Rosemond,E., Roder,J.C., Phillips,A.G., Rubin,E.M., Hersch,S.M., and Hayden,M.R. (1999b). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. Neuron. *23*, 181-192.

Hollenbeck,P.J. and Saxton,W.M. (2005). The axonal transport of mitochondria. J. Cell. Sci. *118*, 5411-5419.

Holmberg,C.I., Staniszewski,K.E., Mensah,K.N., Matouschek,A., and Morimoto,R.I. (2004). Inefficient degradation of truncated polyglutamine proteins by the proteasome. EMBO. J. *23*, 4307-4318.

Huang,T.T. and D'Andrea,A.D. (2006). HAUSP hunting the FOX(O). Nat. Cell. Biol. *8*, 1043-1045.

Iannicola,C., Moreno,S., Oliverio,S., Nardacci,R., Ciofi-Luzzatto,A., and Piacentini,M. (2000). Early alterations in gene expression and cell morphology in a mouse model of Huntington's disease. J. Neurochem. *75*, 830-839.

Ishihara,N., Eura,Y., and Mihara,K. (2004). Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. *117*, 6535-6546.

Ishihara,N., Fujita,Y., Oka,T., and Mihara,K. (2006). Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO. J. *25*, 2966-2977.

Ishihara,N., Nomura,M., Jofuku,A., Kato,H., Suzuki,S.O., Masuda,K., Otera,H., Nakanishi,Y., Nonaka,I., Goto,Y., Taguchi,N., Morinaga,H., Maeda,M., Takayanagi,R., Yokota,S., and Mihara,K. (2009). Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat. Cell. Biol. *11*, 958-966.

Jacobs,H.T., Lehtinen,S.K., and Spelbrink,J.N. (2000). No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. Bioessays. *22*, 564-572.

James,D.I., Parone,P.A., Mattenberger,Y., and Martinou,J.C. (2003). hFis1, a novel component of the mammalian mitochondrial fission machinery. J. Biol. Chem. *278*, 36373-36379.

Jana,N.R., Zemskov,E.A., Wang,G., and Nukina,N. (2001a). Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum. Mol. Genet. *10*, 1049-1059.

Jana,N.R., Zemskov,E.A., Wang,G., and Nukina,N. (2001b). Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum. Mol. Genet. *10*, 1049-1059.

Jenkins,B.G., Koroshetz,W.J., Beal,M.F., and Rosen,B.R. (1993a). Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized 1H NMR spectroscopy. Neurology. *43*, 2689-2695.

Jenkins,B.G., Koroshetz,W.J., Beal,M.F., and Rosen,B.R. (1993b). Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized 1H NMR spectroscopy. Neurology. *43*, 2689-2695.

John,G.B., Shang,Y., Li,L., Renken,C., Mannella,C.A., Selker,J.M., Rangell,L., Bennett,M.J., and Zha,J. (2005). The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol. Biol. Cell *16*, 1543-1554.

Johnson,E.S. (2004). Protein modification by SUMO. Annu. Rev. Biochem. *73*, 355-382.

Jonas,E. (2006). BCL-xL regulates synaptic plasticity. Mol. Interv. *6*, 208-222.

Jones,B.A. and Fangman,W.L. (1992). Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin. Genes. Dev. *6*, 380-389.

Jouaville,L.S., Ichas,F., Holmuhamedov,E.L., Camacho,P., and Lechleiter,J.D. (1995). Synchronization of calcium waves by mitochondrial substrates in Xenopus laevis oocytes. Nature *377*, 438-441.

Kalchman,M.A., Koide,H.B., McCutcheon,K., Graham,R.K., Nichol,K., Nishiyama,K., Kazemi-Esfarjani,P., Lynn,F.C., Wellington,C., Metzler,M., Goldberg,Y.P., Kanazawa,I., Gietz,R.D., and Hayden,M.R. (1997). HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. Nat. Genet. *16*, 44-53.

Kaltenbach,L.S., Romero,E., Becklin,R.R., Chettier,R., Bell,R., Phansalkar,A., Strand,A., Torcassi,C., Savage,J., Hurlburt,A., Cha,G.H., Ukani,L., Chepanoske,C.L., Zhen,Y., Sahasrabudhe,S., Olson,J., Kurschner,C., Ellerby,L.M., Peltier,J.M., Botas,J., and Hughes,R.E. (2007). Huntingtin interacting proteins are genetic modifiers of neurodegeneration. PLoS. Genet. *3*, e82.

Kang,D., Kim,S.H., and Hamasaki,N. (2007). Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. Mitochondrion. *7*, 39-44.

Kanki,T., Nakayama,H., Sasaki,N., Takio,K., Alam,T.I., Hamasaki,N., and Kang,D. (2004). Mitochondrial nucleoid and transcription factor A. Ann. N. Y. Acad. Sci. *1011*, 61-68.

Karbowski,M., Jeong,S.Y., and Youle,R.J. (2004). Endophilin B1 is required for the maintenance of mitochondrial morphology. J. Cell Biol. *166*, 1027-1039.

Karbowski,M., Neutzner,A., and Youle,R.J. (2007). The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J. Cell. Biol. *178*, 71-84.

Karbowski,M., Norris,K.L., Cleland,M.M., Jeong,S.Y., and Youle,R.J. (2006). Role of Bax and Bak in mitochondrial morphogenesis. Nature. *443*, 658-662.

Karbowski,M. and Youle,R.J. (2003). Dynamics of mitochondrial morphology in healthy cells and during apoptosis. Cell Death. Differ. *10*, 870-880.

Kay,B.K., Williamson,M.P., and Sudol,M. (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB. J. *14*, 231-241.

Kegel,K.B., Meloni,A.R., Yi,Y., Kim,Y.J., Doyle,E., Cuiffo,B.G., Sapp,E., Wang,Y., Qin,Z.H., Chen,J.D., Nevins,J.R., Aronin,N., and DiFiglia,M. (2002). Huntingtin is

present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. J. Biol. Chem. *277*, 7466-7476.

Kennedy,L. and Shelbourne,P.F. (2000). Dramatic mutation instability in HD mouse striatum: does polyglutamine load contribute to cell-specific vulnerability in Huntington's disease? Hum. Mol. Genet. *9*, 2539-2544.

Kerr,J.F., Wyllie,A.H., and Currie,A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. *26*, 239-257.

Kiechle,T., Dedeoglu,A., Kubilus,J., Kowall,N.W., Beal,M.F., Friedlander,R.M., Hersch,S.M., and Ferrante,R.J. (2002). Cytochrome C and caspase-9 expression in Huntington's disease. Neuromolecular. Med. *1*, 183-195.

Kijima,K., Numakura,C., Izumino,H., Umetsu,K., Nezu,A., Shiiki,T., Ogawa,M., Ishizaki,Y., Kitamura,T., Shozawa,Y., and Hayasaka,K. (2005). Mitochondrial GTPase mitofusin 2 mutation in Charcot-Marie-Tooth neuropathy type 2A. Hum. Genet. *116*, 23-27.

Kim,G.W. and Chan,P.H. (2001). Oxidative stress and neuronal DNA fragmentation mediate age-dependent vulnerability to the mitochondrial toxin, 3-nitropropionic acid, in the mouse striatum. Neurobiol. Dis. *8*, 114-126.

Kim,M., Lee,H.S., LaForet,G., McIntyre,C., Martin,E.J., Chang,P., Kim,T.W., Williams,M., Reddy,P.H., Tagle,D., Boyce,F.M., Won,L., Heller,A., Aronin,N., and DiFiglia,M. (1999). Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. J. Neurosci. *19*, 964-973.

Kim,Y.J., Yi,Y., Sapp,E., Wang,Y., Cuiffo,B., Kegel,K.B., Qin,Z.H., Aronin,N., and DiFiglia,M. (2001). Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc. Natl. Acad. Sci. U. S. A. *98*, 12784-12789.

Kirkwood,S.C., Su,J.L., Conneally,P., and Foroud,T. (2001). Progression of symptoms in the early and middle stages of Huntington disease. Arch. Neurol. *58*, 273-278.

Kjer,B., Eiberg,H., Kjer,P., and Rosenberg,T. (1996). Dominant optic atrophy mapped to chromosome 3q region. II. Clinical and epidemiological aspects. Acta. Ophthalmol. Scand. *74*, 3-7.

Koch,A., Thiemann,M., Grabenbauer,M., Yoon,Y., McNiven,M.A., and Schrader,M. (2003). Dynamin-like protein 1 is involved in peroxisomal fission. J. Biol. Chem. *278*, 8597-8605.

Koch,A., Yoon,Y., Bonekamp,N.A., McNiven,M.A., and Schrader,M. (2005). A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Mol. Biol. Cell. *16*, 5077-5086.

Koehler,C.M. (2004). New developments in mitochondrial assembly. Annu. Rev. Cell. Dev. Biol. *20*, 309-335.

Koehler,C.M., Leuenberger,D., Merchant,S., Renold,A., Junne,T., and Schatz,G. (1999). Human deafness dystonia syndrome is a mitochondrial disease. Proc. Natl. Acad. Sci. U. S. A. *96*, 2141-2146.

Koroshetz,W.J., Jenkins,B.G., Rosen,B.R., and Beal,M.F. (1997). Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. Ann. Neurol. *41*, 160-165.

Koshiba,T., Detmer,S.A., Kaiser,J.T., Chen,H., McCaffery,J.M., and Chan,D.C. (2004). Structural basis of mitochondrial tethering by mitofusin complexes. Science *305*, 858-862.

Krammer,P.H. (2000). CD95's deadly mission in the immune system. Nature. *407*, 789-795.

Kremer,B., Goldberg,P., Andrew,S.E., Theilmann,J., Telenius,H., Zeisler,J., Squitieri,F., Lin,B., Bassett,A., Almqvist,E., and . (1994). A worldwide study of the Huntington's disease mutation. The sensitivity and specificity of measuring CAG repeats. N. Engl. J. Med. *330*, 1401-1406.

Kroemer,G. and Zitvogel,L. (2007). Death, danger, and immunity: an infernal trio. Immunol. Rev. *220*, 5-7.

Kuhl,D.E., Phelps,M.E., Markham,C.H., Metter,E.J., Riege,W.H., and Winter,J. (1982). Cerebral metabolism and atrophy in Huntington's disease determined by 18FDG and computed tomographic scan. Ann. Neurol. *12*, 425-434.

Labrousse,A.M., Zappaterra,M.D., Rube,D.A., and van der Bliek,A.M. (1999). C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol. Cell *4*, 815-826.

Laforet,G.A., Sapp,E., Chase,K., McIntyre,C., Boyce,F.M., Campbell,M., Cadigan,B.A., Warzecki,L., Tagle,D.A., Reddy,P.H., Cepeda,C., Calvert,C.R., Jokel,E.S., Klapstein,G.J., Ariano,M.A., Levine,M.S., DiFiglia,M., and Aronin,N. (2001). Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J. Neurosci. *21*, 9112-9123.

Langer,T., Kaser,M., Klanner,C., and Leonhard,K. (2001). AAA proteases of mitochondria: quality control of membrane proteins and regulatory functions during mitochondrial biogenesis. Biochem. Soc. Trans. *29*, 431-436.

Lawson,V.H., Graham,B.V., and Flanigan,K.M. (2005). Clinical and electrophysiologic features of CMT2A with mutations in the mitofusin 2 gene. Neurology. *65*, 197-204.

Lee,H.C. and Wei,Y.H. (2000). Mitochondrial role in life and death of the cell. J. Biomed. Sci. *7*, 2-15.

Lee,Y.J., Jeong,S.Y., Karbowski,M., Smith,C.L., and Youle,R.J. (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol. Biol. Cell *15*, 5001-5011.

Leeflang,E.P., Tavare,S., Marjoram,P., Neal,C.O., Srinidhi,J., MacFarlane,H., MacDonald,M.E., Gusella,J.F., de Young,M., Wexler,N.S., and Arnheim,N. (1999). Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. Hum. Mol. Genet. *8*, 173-183.

Leenders,K.L., Frackowiak,R.S., Quinn,N., and Marsden,C.D. (1986). Brain energy metabolism and dopaminergic function in Huntington's disease measured in vivo using positron emission tomography. Mov. Disord. *1*, 69-77.

Legros,F., Lombes,A., Frachon,P., and Rojo,M. (2002). Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. Mol. Biol. Cell *13*, 4343-4354.

Legros,F., Malka,F., Frachon,P., Lombes,A., and Rojo,M. (2004). Organization and dynamics of human mitochondrial DNA. J. Cell. Sci. *117*, 2653-2662.

Lesage,S., Condroyer,C., Lannuzel,A., Lohmann,E., Troiano,A., Tison,F., Damier,P., Thobois,S., Ouvrard-Hernandez,A.M., Rivaud-Pechoux,S., Brefel-Courbon,C., Destee,A., Tranchant,C., Romana,M., Leclere,L., Durr,A., Brice,A., and French Parkinson's Disease Genetics Study Group (2009). Molecular analyses of the LRRK2 gene in European and North African autosomal dominant Parkinson's disease. J. Med. Genet. *46*, 458-464.

Levine,M.S., Klapstein,G.J., Koppel,A., Gruen,E., Cepeda,C., Vargas,M.E., Jokel,E.S., Carpenter,E.M., Zanjani,H., Hurst,R.S., Efstratiadis,A., Zeitlin,S., and Chesselet,M.F. (1999). Enhanced sensitivity to N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. J. Neurosci. Res. *58*, 515-532.

Li,H., Chen,Y., Jones,A.F., Sanger,R.H., Collis,L.P., Flannery,R., McNay,E.C., Yu,T., Schwarzenbacher,R., Bossy,B., Bossy-Wetzel,E., Bennett,M.V., Pypaert,M., Hickman,J.A., Smith,P.J., Hardwick,J.M., and Jonas,E.A. (2008). Bcl-xL induces Drp1-dependent synapse formation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U. S. A. *105*, 2169-2174.

Li,H., Li,S.H., Johnston,H., Shelbourne,P.F., and Li,X.J. (2000). Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. Nat. Genet. *25*, 385-389.

Li,S.H., Cheng,A.L., Zhou,H., Lam,S., Rao,M., Li,H., and Li,X.J. (2002). Interaction of Huntington disease protein with transcriptional activator Sp1. Mol. Cell. Biol. *22*, 1277-1287.

Li,S.H., Gutekunst,C.A., Hersch,S.M., and Li,X.J. (1998a). Association of HAP1 isoforms with a unique cytoplasmic structure. J. Neurochem. *71*, 2178-2185.

Li,S.H., Gutekunst,C.A., Hersch,S.M., and Li,X.J. (1998b). Association of HAP1 isoforms with a unique cytoplasmic structure. J. Neurochem. *71*, 2178-2185.

Li,X. and Gould,S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. J. Biol. Chem. *278*, 17012-17020.

Li,X.J. and Li,S.H. (2005). HAP1 and intracellular trafficking. Trends. Pharmacol. Sci. *26*, 1-3.

Li,X.J., Li,S.H., Sharp,A.H., Nucifora,F.C., Jr., Schilling,G., Lanahan,A., Worley,P., Snyder,S.H., and Ross,C.A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. Nature. *378*, 398-402.

Li,Z., Okamoto,K., Hayashi,Y., and Sheng,M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell *119*, 873-887.

Lin,C.H., Tallaksen-Greene,S., Chien,W.M., Cearley,J.A., Jackson,W.S., Crouse,A.B., Ren,S., Li,X.J., Albin,R.L., and Detloff,P.J. (2001). Neurological abnormalities in a knock-in mouse model of Huntington's disease. Hum. Mol. Genet. *10*, 137-144.

Liot,G., Bossy,B., Lubitz,S., Kushnareva,Y., Sejbuk,N., and Bossy-Wetzel,E. (2009). Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an. Cell. Death. Differ. *16*, 899-909.

Liu,X., Kim,C.N., Yang,J., Jemmerson,R., and Wang,X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Liu,Y.F. (1998). Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. J. Biol. Chem. *273*, 28873-28877.

Lodi,R., Schapira,A.H., Manners,D., Styles,P., Wood,N.W., Taylor,D.J., and Warner,T.T. (2000). Abnormal in vivo skeletal muscle energy metabolism in Huntington's disease and dentatorubropallidoluysian atrophy. Ann. Neurol. *48*, 72-76.

Lodi,R., Tonon,C., Valentino,M.L., Iotti,S., Clementi,V., Malucelli,E., Barboni,P., Longanesi,L., Schimpf,S., Wissinger,B., Baruzzi,A., Barbiroli,B., and Carelli,V. (2004). Deficit of in vivo mitochondrial ATP production in OPA1-related dominant optic atrophy. Ann. Neurol. *56*, 719-723.

LUCAS,D.R. and NEWHOUSE,J.P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. AMA. Arch. Ophthalmol. *58*, 193-201.

Lutz,A.K., Exner,N., Fett,M.E., Schlehe,J.S., Kloos,K., Lammermann,K., Brunner,B., Kurz-Drexler,A., Vogel,F., Reichert,A.S., Bouman,L., Vogt-Weisenhorn,D., Wurst,W., Tatzelt,J., Haass,C., and Winklhofer,K.F. (2009). Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. J. Biol. Chem. *284*, 22938-22951.

Malka,F., Lombes,A., and Rojo,M. (2006). Organization, dynamics and transmission of mitochondrial DNA: focus on vertebrate nucleoids. Biochim. Biophys. Acta. *1763*, 463-472.

Mandemakers,W., Morais,V.A., and De Strooper,B. (2007). A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases. J. Cell. Sci. *120*, 1707-1716.

Mannella,C.A., Marko,M., Penczek,P., Barnard,D., and Frank,J. (1994). The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope. Microsc. Res. Tech. *27*, 278-283.

Marchbank,N.J., Craig,J.E., Leek,J.P., Toohey,M., Churchill,A.J., Markham,A.F., Mackey,D.A., Toomes,C., and Inglehearn,C.F. (2002). Deletion of the OPA1 gene in a dominant optic atrophy family: evidence that haploinsufficiency is the cause of disease. J. Med. Genet. *39*, e47.

Margulis,L. (1971). The origin of plant and animal cells. Am. Sci. *59*, 230-235.

Martin,J.B. and Gusella,J.F. (1986). Huntington's disease. Pathogenesis and management. N. Engl. J. Med. *315*, 1267-1276.

Mattenberger,Y., James,D.I., and Martinou,J.C. (2003). Fusion of mitochondria in mammalian cells is dependent on the mitochondrial inner membrane potential and independent of microtubules or actin. FEBS Lett. *538*, 53-59.

Matthews,R.T., Yang,L., Jenkins,B.G., Ferrante,R.J., Rosen,B.R., Kaddurah-Daouk,R., and Beal,M.F. (1998). Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. J. Neurosci. *18*, 156-163.

McNiven,M.A., Cao,H., Pitts,K.R., and Yoon,Y. (2000). The dynamin family of mechanoenzymes: pinching in new places. Trends Biochem. Sci. *25*, 115-120.

McQuibban,G.A., Saurya,S., and Freeman,M. (2003). Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature *423*, 537-541.

Meeusen,S., McCaffery,J.M., and Nunnari,J. (2004). Mitochondrial fusion intermediates revealed in vitro. Science. *305*, 1747-1752.

Meier,P., Finch,A., and Evan,G. (2000). Apoptosis in development. Nature. *407*, 796-801.

Merkwirth,C., Dargazanli,S., Tatsuta,T., Geimer,S., Lower,B., Wunderlich,F.T., Kleist-Retzow,J.C., Waisman,A., Westermann,B., and Langer,T. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes. Dev. *22*, 476-488.

Metzler,M., Helgason,C.D., Dragatsis,I., Zhang,T., Gan,L., Pineault,N., Zeitlin,S.O., Humphries,R.K., and Hayden,M.R. (2000). Huntingtin is required for normal hematopoiesis. Hum. Mol. Genet. *9*, 387-394.

Metzler,M., Legendre-Guillemin,V., Gan,L., Chopra,V., Kwok,A., McPherson,P.S., and Hayden,M.R. (2001). HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. J. Biol. Chem. *276*, 39271-39276.

Minauro-Sanmiguel,F., Wilkens,S., and Garcia,J.J. (2005). Structure of dimeric mitochondrial ATP synthase: novel F0 bridging features and the structural basis of mitochondrial cristae biogenesis. Proc. Natl. Acad. Sci. U. S. A. *102*, 12356-12358.

Misaka,T., Miyashita,T., and Kubo,Y. (2002). Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. J. Biol. Chem. *277*, 15834-15842.

Mitchell,P. (1979). Keilin's respiratory chain concept and its chemiosmotic consequences. Science. *206*, 1148-1159.

Miyashita,T. and Reed,J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. *80*, 293-299.

Mootha,V.K., Wei,M.C., Buttle,K.F., Scorrano,L., Panoutsakopoulou,V., Mannella,C.A., and Korsmeyer,S.J. (2001). A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. EMBO J. *20*, 661-671.

Mopert,K., Hajek,P., Frank,S., Chen,C., Kaufmann,J., and Santel,A. (2009). Loss of Drp1 function alters OPA1 processing and changes mitochondrial membrane organization. Exp. Cell. Res. *315*, 2165-2180.

Mormone,E., Matarrese,P., Tinari,A., Cannella,M., Maglione,V., Farrace,M.G., Piacentini,M., Frati,L., Malorni,W., and Squitieri,F. (2006). Genotype-dependent priming to self- and xeno-cannibalism in heterozygous and homozygous lymphoblasts from patients with Huntington's disease. J. Neurochem. *98*, 1090-1099.

Morris,R.L. and Hollenbeck,P.J. (1995). Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons. J. Cell. Biol. *131*, 1315-1326.

Mortiboys,H., Thomas,K.J., Koopman,W.J., Klaffke,S., Abou-Sleiman,P., Olpin,S., Wood,N.W., Willems,P.H., Smeitink,J.A., Cookson,M.R., and Bandmann,O. (2008). Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. Ann. Neurol. *64*, 555-565.

Nagai,Y., Tucker,T., Ren,H., Kenan,D.J., Henderson,B.S., Keene,J.D., Strittmatter,W.J., and Burke,J.R. (2000). Inhibition of polyglutamine protein aggregation and cell death by novel peptides identified by phage display screening. J. Biol. Chem. *275*, 10437-10442.

Nakada,K., Inoue,K., and Hayashi,J.I. (2001). Mito-mice: animal models for mitochondrial DNA-based diseases. Semin. Cell. Dev. Biol. *12*, 459-465.

Nakamura,N., Kimura,Y., Tokuda,M., Honda,S., and Hirose,S. (2006). MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBO. Rep. *7*, 1019-1022.

Narendra,D., Tanaka,A., Suen,D.F., and Youle,R.J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell. Biol. *183*, 795-803.

Neutzner,A. and Youle,R.J. (2005). Instability of the mitofusin Fzo1 regulates mitochondrial morphology during the mating response of the yeast Saccharomyces cerevisiae. J. Biol. Chem. *280*, 18598-18603.

Neutzner,A., Youle,R.J., and Karbowski,M. (2007). Outer mitochondrial membrane protein degradation by the proteasome. Novartis. Found. Symp. *287*, 4-14.

Nosek,J. and Tomaska,L. (2003). Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. Curr. Genet. *44*, 73-84.

Novelli,A., Reilly,J.A., Lysko,P.G., and Henneberry,R.C. (1988). Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain. Res. *451*, 205-212.

Nucifora,F.C., Jr., Sasaki,M., Peters,M.F., Huang,H., Cooper,J.K., Yamada,M., Takahashi,H., Tsuji,S., Troncoso,J., Dawson,V.L., Dawson,T.M., and Ross,C.A. (2001). Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science. *291*, 2423-2428.

Olichon,A., Baricault,L., Gas,N., Guillou,E., Valette,A., Belenguer,P., and Lenaers,G. (2003). Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J. Biol. Chem. *278*, 7743-7746.

Ona,V.O., Li,M., Vonsattel,J.P., Andrews,L.J., Khan,S.Q., Chung,W.M., Frey,A.S., Menon,A.S., Li,X.J., Stieg,P.E., Yuan,J., Penney,J.B., Young,A.B., Cha,J.H., and Friedlander,R.M. (1999). Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. Nature. *399*, 263-267.

Ono,T., Isobe,K., Nakada,K., and Hayashi,J.I. (2001). Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. Nat. Genet. *28*, 272-275.

Orr,A.L., Li,S., Wang,C.E., Li,H., Wang,J., Rong,J., Xu,X., Mastroberardino,P.G., Greenamyre,J.T., and Li,X.J. (2008a). N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. J. Neurosci. *28*, 2783-2792.

Orr,A.L., Li,S., Wang,C.E., Li,H., Wang,J., Rong,J., Xu,X., Mastroberardino,P.G., Greenamyre,J.T., and Li,X.J. (2008b). N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. J. Neurosci. *28*, 2783-2792.

Ott,M., Robertson,J.D., Gogvadze,V., Zhivotovsky,B., and Orrenius,S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. Proc. Natl. Acad. Sci. U. S. A *99*, 1259-1263.

Pacher,P. and Hajnoczky,G. (2001). Propagation of the apoptotic signal by mitochondrial waves. EMBO J. *20*, 4107-4121.

Palade,G.E. (1952). The fine structure of mitochondria. Anat. Rec. *114*, 427-451.

Panov,A.V., Gutekunst,C.A., Leavitt,B.R., Hayden,M.R., Burke,J.R., Strittmatter,W.J., and Greenamyre,J.T. (2002a). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat. Neurosci. *5*, 731-736.

Panov,A.V., Gutekunst,C.A., Leavitt,B.R., Hayden,M.R., Burke,J.R., Strittmatter,W.J., and Greenamyre,J.T. (2002b). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat. Neurosci. *5*, 731-736.

Park,J., Lee,G., and Chung,J. (2009a). The PINK1-Parkin pathway is involved in the regulation of mitochondrial remodeling process. Biochem. Biophys. Res. Commun. *378*, 518-523.

Park,J., Lee,G., and Chung,J. (2009b). The PINK1-Parkin pathway is involved in the regulation of mitochondrial remodeling process. Biochem. Biophys. Res. Commun. *378*, 518-523.

Park,J., Lee,S.B., Lee,S., Kim,Y., Song,S., Kim,S., Bae,E., Kim,J., Shong,M., Kim,J.M., and Chung,J. (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature. *441*, 1157-1161.

Parone,P.A., Da Cruz,S., Tondera,D., Mattenberger,Y., James,D.I., Maechler,P., Barja,F., and Martinou,J.C. (2008a). Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS. One. *3*, e3257.

Parone,P.A., Da Cruz,S., Tondera,D., Mattenberger,Y., James,D.I., Maechler,P., Barja,F., and Martinou,J.C. (2008b). Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS. One. *3*, e3257.

Paumard,P., Vaillier,J., Coulary,B., Schaeffer,J., Soubannier,V., Mueller,D.M., Brethes,D., di Rago,J.P., and Velours,J. (2002). The ATP synthase is involved in generating mitochondrial cristae morphology. EMBO J. *21*, 221-230.

Pellegrini,L., Passer,B.J., Canelles,M., Lefterov,I., Ganjei,J.K., Fowlkes,B.J., Koonin,E.V., and D'Adamio,L. (2001). PAMP and PARL, two novel putative metalloproteases interacting with the COOH-terminus of Presenilin-1 and -2. J. Alzheimers. Dis. *3*, 181-190.

Pelloquin,L., Belenguer,P., Menon,Y., Gas,N., and Ducommun,B. (1999). Fission yeast Msp1 is a mitochondrial dynamin-related protein. J. Cell. Sci. *112 (Pt 22)*, 4151-4161.

Perkins,G.A., Renken,C.W., Frey,T.G., and Ellisman,M.H. (2001). Membrane architecture of mitochondria in neurons of the central nervous system. J. Neurosci. Res. *66*, 857-865.

Perotti,M.E., Anderson,W.A., and Swift,H. (1983). Quantitative cytochemistry of the diaminobenzidine cytochrome oxidase reaction product in mitochondria of cardiac muscle and pancreas. J. Histochem. Cytochem. *31*, 351-365.

Pesch,U.E., Leo-Kottler,B., Mayer,S., Jurklies,B., Kellner,U., Apfelstedt-Sylla,E., Zrenner,E., Alexander,C., and Wissinger,B. (2001). OPA1 mutations in patients with autosomal dominant optic atrophy and evidence for semi-dominant inheritance. Hum. Mol. Genet. *10*, 1359-1368.

Peters,M.F., Nucifora,F.C., Jr., Kushi,J., Seaman,H.C., Cooper,J.K., Herring,W.J., Dawson,V.L., Dawson,T.M., and Ross,C.A. (1999). Nuclear targeting of mutant Huntingtin increases toxicity. Mol. Cell. Neurosci. *14*, 121-128.

Polidori,M.C., Mecocci,P., Browne,S.E., Senin,U., and Beal,M.F. (1999). Oxidative damage to mitochondrial DNA in Huntington's disease parietal cortex. Neurosci. Lett. *272*, 53-56.

Poole,A.C., Thomas,R.E., Andrews,L.A., McBride,H.M., Whitworth,A.J., and Pallanck,L.J. (2008). The PINK1/Parkin pathway regulates mitochondrial morphology. Proc. Natl. Acad. Sci. U. S. A. *105*, 1638-1643.

Portera-Cailliau,C., Hedreen,J.C., Price,D.L., and Koliatsos,V.E. (1995a). Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. J. Neurosci. *15*, 3775-3787.

Portera-Cailliau,C., Hedreen,J.C., Price,D.L., and Koliatsos,V.E. (1995b). Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. J. Neurosci. *15*, 3775-3787.

Poyton,R.O. and McEwen,J.E. (1996). Crosstalk between nuclear and mitochondrial genomes. Annu. Rev. Biochem. *65*, 563-607.

Prokisch,H., Scharfe,C., Camp,D.G., Xiao,W., David,L., Andreoli,C., Monroe,M.E., Moore,R.J., Gritsenko,M.A., Kozany,C., Hixson,K.K., Mottaz,H.M., Zischka,H., Ueffing,M., Herman,Z.S., Davis,R.W., Meitinger,T., Oefner,P.J., Smith,R.D., and Steinmetz,L.M. (2004). Integrative analysis of the mitochondrial proteome in yeast. PLoS. Biol. *2*, e160.

Puigserver,P. and Spiegelman,B.M. (2003). Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr. Rev. *24*, 78-90.

Purdon,S.E., Mohr,E., Ilivitsky,V., and Jones,B.D. (1994). Huntington's disease: pathogenesis, diagnosis and treatment. J. Psychiatry. Neurosci. *19*, 359-367.

Rapaport,D., Brunner,M., Neupert,W., and Westermann,B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. J. Biol. Chem. *273*, 20150-20155.

Ravikumar,B., Duden,R., and Rubinsztein,D.C. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum. Mol. Genet. *11*, 1107-1117.

Ravikumar,B., Vacher,C., Berger,Z., Davies,J.E., Luo,S., Oroz,L.G., Scaravilli,F., Easton,D.F., Duden,R., O'Kane,C.J., and Rubinsztein,D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. *36*, 585-595.

Reddy,P.H., Williams,M., Charles,V., Garrett,L., Pike-Buchanan,L., Whetsell,W.O., Jr., Miller,G., and Tagle,D.A. (1998). Behavioural abnormalities and selective neuronal

loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat. Genet. *20*, 198-202.

Rikhy,R., Kamat,S., Ramagiri,S., Sriram,V., and Krishnan,K.S. (2007). Mutations in dynamin-related protein result in gross changes in mitochondrial morphology and affect synaptic vesicle recycling at the Drosophila neuromuscular junction. Genes. Brain. Behav. *6*, 42-53.

Rizzuto,R., Bernardi,P., and Pozzan,T. (2000). Mitochondria as all-round players of the calcium game. J. Physiol. *529 Pt 1:37-47.*, 37-47.

Rodriguez,J. and Lazebnik,Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. Genes. Dev. *13*, 3179-3184.

Rojo,M., Legros,F., Chateau,D., and Lombes,A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. J. Cell Sci. *115*, 1663-1674.

Rothman,S.M. and Olney,J.W. (1995). Excitotoxicity and the NMDA receptor--still lethal after eight years. Trends. Neurosci. *18*, 57-58.

Rubinsztein,D.C. (2006a). The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. *443*, 780-786.

Rubinsztein,D.C. (2006b). The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. *443*, 780-786.

Saft,C., Zange,J., Andrich,J., Muller,K., Lindenberg,K., Landwehrmeyer,B., Vorgerd,M., Kraus,P.H., Przuntek,H., and Schols,L. (2005). Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. Mov. Disord. *20*, 674-679.

Sanberg,P.R., Calderon,S.F., Giordano,M., Tew,J.M., and Norman,A.B. (1989). The quinolinic acid model of Huntington's disease: locomotor abnormalities. Exp. Neurol. *105*, 45-53.

Sanberg,P.R., Fibiger,H.C., and Mark,R.F. (1981). Body weight and dietary factors in Huntington's disease patients compared with matched controls. Med. J. Aust. *1*, 407-409.

Sanchez,I., Xu,C.J., Juo,P., Kakizaka,A., Blenis,J., and Yuan,J. (1999). Caspase-8 is required for cell death induced by expanded polyglutamine repeats. Neuron. *22*, 623-633.

Sandebring,A., Thomas,K.J., Beilina,A., van der,B.M., Cleland,M.M., Ahmad,R., Miller,D.W., Zambrano,I., Cowburn,R.F., Behbahani,H., Cedazo-Minguez,A., and Cookson,M.R. (2009). Mitochondrial alterations in PINK1 deficient cells are influenced by calcineurin-dependent dephosphorylation of dynamin-related protein 1. PLoS. One. *4*, e5701.

Santel,A., Frank,S., Gaume,B., Herrler,M., Youle,R.J., and Fuller,M.T. (2003). Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. J. Cell Sci. *116*, 2763-2774.

Santel,A. and Fuller,M.T. (2001). Control of mitochondrial morphology by a human mitofusin. J. Cell Sci. *114*, 867-874.

Sapp,E., Penney,J., Young,A., Aronin,N., Vonsattel,J.P., and DiFiglia,M. (1999). Axonal transport of N-terminal huntingtin suggests early pathology of corticostriatal projections in Huntington disease. J. Neuropathol. Exp. Neurol. *58*, 165-173.

Sapp,E., Schwarz,C., Chase,K., Bhide,P.G., Young,A.B., Penney,J., Vonsattel,J.P., Aronin,N., and DiFiglia,M. (1997). Huntingtin localization in brains of normal and Huntington's disease patients. Ann. Neurol. *42*, 604-612.

Satoh,M., Hamamoto,T., Seo,N., Kagawa,Y., and Endo,H. (2003). Differential sublocalization of the dynamin-related protein OPA1 isoforms in mitochondria. Biochem. Biophys. Res. Commun. *300*, 482-493.

Saudou,F., Finkbeiner,S., Devys,D., and Greenberg,M.E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell. *95*, 55-66.

Sawa,A., Wiegand,G.W., Cooper,J., Margolis,R.L., Sharp,A.H., Lawler,J.F., Jr., Greenamyre,J.T., Snyder,S.H., and Ross,C.A. (1999). Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. Nat. Med. *5*, 1194-1198.

Schapira,A.H. (2006). Mitochondrial disease. Lancet. *368*, 70-82.

Scherzinger,E., Lurz,R., Turmaine,M., Mangiarini,L., Hollenbach,B., Hasenbank,R., Bates,G.P., Davies,S.W., Lehrach,H., and Wanker,E.E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell. *90*, 549-558.

Schilling,G., Becher,M.W., Sharp,A.H., Jinnah,H.A., Duan,K., Kotzuk,J.A., Slunt,H.H., Ratovitski,T., Cooper,J.K., Jenkins,N.A., Copeland,N.G., Price,D.L., Ross,C.A., and Borchelt,D.R. (1999). Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum. Mol. Genet. *8*, 397-407.

Schilling,G., Sharp,A.H., Loev,S.J., Wagster,M.V., Li,S.H., Stine,O.C., and Ross,C.A. (1995). Expression of the Huntington's disease (IT15) protein product in HD patients. Hum. Mol. Genet. *4*, 1365-1371.

Schmidt,A., Wolde,M., Thiele,C., Fest,W., Kratzin,H., Podtelejnikov,A.V., Witke,W., Huttner,W.B., and Soling,H.D. (1999). Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature. *401*, 133-141.

Schwarcz,R., Foster,A.C., French,E.D., Whetsell,W.O., Jr., and Kohler,C. (1984). Excitotoxic models for neurodegenerative disorders. Life. Sci. *35*, 19-32.

Scorrano,L., Ashiya,M., Buttle,K., Weiler,S., Oakes,S.A., Mannella,C.A., and Korsmeyer,S.J. (2002). A Distinct Pathway Remodels Mitochondrial Cristae and Mobilizes Cytochrome c during Apoptosis. Dev. Cell *2*, 55-67.

Scorrano,L. and Korsmeyer,S.J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochemical and Biophysical Research Communications *304*, 437-444.

Seong,I.S., Ivanova,E., Lee,J.M., Choo,Y.S., Fossale,E., Anderson,M., Gusella,J.F., Laramie,J.M., Myers,R.H., Lesort,M., and MacDonald,M.E. (2005). HD CAG repeat implicates a dominant property of huntingtin in mitochondrial energy metabolism. Hum. Mol. Genet. *14*, 2871-2880.

Sesaki,H., Southard,S.M., Hobbs,A.E., and Jensen,R.E. (2003a). Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion. Biochem. Biophys. Res. Commun. *308*, 276-283.

Sesaki,H., Southard,S.M., Yaffe,M.P., and Jensen,R.E. (2003b). Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. Mol. Biol. Cell *14*, 2342-2356.

Sharp,A.H., Loev,S.J., Schilling,G., Li,S.H., Li,X.J., Bao,J., Wagster,M.V., Kotzuk,J.A., Steiner,J.P., Lo,A., and . (1995). Widespread expression of Huntington's disease gene (IT15) protein product. Neuron. *14*, 1065-1074.

Shaw,J.M. and Nunnari,J. (2002). Mitochondrial dynamics and division in budding yeast. Trends Cell Biol. *12*, 178-184.

Shelbourne,P.F., Killeen,N., Hevner,R.F., Johnston,H.M., Tecott,L., Lewandoski,M., Ennis,M., Ramirez,L., Li,Z., Iannicola,C., Littman,D.R., and Myers,R.M. (1999). A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. Hum. Mol. Genet. *8*, 763-774.

Shimura,H., Hattori,N., Kubo,S., Mizuno,Y., Asakawa,S., Minoshima,S., Shimizu,N., Iwai,K., Chiba,T., Tanaka,K., and Suzuki,T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. *25*, 302-305.

Sickmann,A., Reinders,J., Wagner,Y., Joppich,C., Zahedi,R., Meyer,H.E., Schonfisch,B., Perschil,I., Chacinska,A., Guiard,B., Rehling,P., Pfanner,N., and Meisinger,C. (2003). The proteome of Saccharomyces cerevisiae mitochondria. Proc. Natl. Acad. Sci. U. S. A. *100*, 13207-13212.

Sipione,S. and Cattaneo,E. (2001). Modeling Huntington's disease in cells, flies, and mice. Mol. Neurobiol. *23*, 21-51.

Smirnova,E., Griparic,L., Shurland,D.L., and van der Bliek,A.M. (2001). Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell *12*, 2245-2256.

Song,C., Zhang,Y., Parsons,C.G., and Liu,Y.F. (2003). Expression of polyglutamine-expanded huntingtin induces tyrosine phosphorylation of N-methyl-D-aspartate receptors. J. Biol. Chem. *278*, 33364-33369.

Song,Z., Chen,H., Fiket,M., Alexander,C., and Chan,D.C. (2007). OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J. Cell. Biol. *178*, 749-755.

Spinazzi,M., Cazzola,S., Bortolozzi,M., Baracca,A., Loro,E., Casarin,A., Solaini,G., Sgarbi,G., Casalena,G., Cenacchi,G., Malena,A., Frezza,C., Carrara,F., Angelini,C., Scorrano,L., Salviati,L., and Vergani,L. (2008). A novel deletion in the GTPase domain of OPA1 causes defects in mitochondrial morphology and distribution, but not in function. Hum. Mol. Genet. *17*, 3291-3302.

Squitieri,F., Cannella,M., Sgarbi,G., Maglione,V., Falleni,A., Lenzi,P., Baracca,A., Cislaghi,G., Saft,C., Ragona,G., Russo,M.A., Thompson,L.M., Solaini,G., and Fornai,F. (2006). Severe ultrastructural mitochondrial changes in lymphoblasts homozygous for Huntington disease mutation. Mech. Ageing. Dev. *127*, 217-220.

Squitieri,F., Falleni,A., Cannella,M., Orobello,S., Fulceri,F., Lenzi,P., and Fornai,F. (2009). Abnormal morphology of peripheral cell tissues from patients with Huntington disease. J. Neural. Transm.

Squitieri,F., Gellera,C., Cannella,M., Mariotti,C., Cislaghi,G., Rubinsztein,D.C., Almqvist,E.W., Turner,D., Bachoud-Levi,A.C., Simpson,S.A., Delatycki,M., Maglione,V., Hayden,M.R., and Donato,S.D. (2003). Homozygosity for CAG mutation in Huntington disease is associated with a more severe clinical course. Brain. *126*, 946-955.

Stahl,W.L. and Swanson,P.D. (1974). Biochemical abnormalities in Huntington's chorea brains. Neurology. *24*, 813-819.

Staub,O. and Rotin,D. (2006). Role of ubiquitylation in cellular membrane transport. Physiol. Rev. *86*, 669-707.

Steffan,J.S., Bodai,L., Pallos,J., Poelman,M., McCampbell,A., Apostol,B.L., Kazantsev,A., Schmidt,E., Zhu,Y.Z., Greenwald,M., Kurokawa,R., Housman,D.E., Jackson,G.R., Marsh,J.L., and Thompson,L.M. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature. *413*, 739-743.

Steffan,J.S., Kazantsev,A., Spasic-Boskovic,O., Greenwald,M., Zhu,Y.Z., Gohler,H., Wanker,E.E., Bates,G.P., Housman,D.E., and Thompson,L.M. (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. Proc. Natl. Acad. Sci. U. S. A. *97*, 6763-6768.

Stojanovski,D., Koutsopoulos,O.S., Okamoto,K., and Ryan,M.T. (2004). Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. J. Cell. Sci. *117*, 1201-1210.

Stowers,R.S., Megeath,L.J., Gorska-Andrzejak,J., Meinertzhagen,I.A., and Schwarz,T.L. (2002). Axonal transport of mitochondria to synapses depends on milton, a novel Drosophila protein. Neuron. *36*, 1063-1077.

Sugars,K.L. and Rubinsztein,D.C. (2003). Transcriptional abnormalities in Huntington disease. Trends. Genet. *19*, 233-238.

Sugioka,R., Shimizu,S., and Tsujimoto,Y. (2004). Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J. Biol. Chem. *279*, 52726-52734.

Sun,Y., Savanenin,A., Reddy,P.H., and Liu,Y.F. (2001). Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. J. Biol. Chem. *276*, 24713-24718.

Suzuki,M., Jeong,S.Y., Karbowski,M., Youle,R.J., and Tjandra,N. (2003). The solution structure of human mitochondria fission protein Fis1 reveals a novel TPR-like helix bundle. J. Mol. Biol. *334*, 445-458.

Szabadkai,G., Simoni,A.M., Chami,M., Wieckowski,M.R., Youle,R.J., and Rizzuto,R. (2004). Drp-1-Dependent Division of the Mitochondrial Network Blocks Intraorganellar Ca(2+) Waves and Protects against Ca(2+)-Mediated Apoptosis. Mol. Cell *16*, 59-68.

Szebenyi,G., Morfini,G.A., Babcock,A., Gould,M., Selkoe,K., Stenoien,D.L., Young,M., Faber,P.W., MacDonald,M.E., McPhaul,M.J., and Brady,S.T. (2003). Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. Neuron. *40*, 41-52.

Tabrizi,S.J., Cleeter,M.W., Xuereb,J., Taanman,J.W., Cooper,J.M., and Schapira,A.H. (1999). Biochemical abnormalities and excitotoxicity in Huntington's disease brain. Ann. Neurol. *45*, 25-32.

Taguchi,N., Ishihara,N., Jofuku,A., Oka,T., and Mihara,K. (2007). Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. J. Biol. Chem. *282*, 11521-11529.

Takano,H. and Gusella,J.F. (2002). The predominantly HEAT-like motif structure of huntingtin and its association and coincident nuclear entry with dorsal, an NF-kB/Rel/dorsal family transcription factor. BMC. Neurosci. *3*, 15.

Tellez-Nagel,I., Johnson,A.B., and Terry,R.D. (1974). Studies on brain biopsies of patients with Huntington's chorea. J. Neuropathol. Exp. Neurol. *33*, 308-332.

Thomas,L.B., Gates,D.J., Richfield,E.K., O'Brien,T.F., Schweitzer,J.B., and Steindler,D.A. (1995). DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. Exp. Neurol. *133*, 265-272.

Tolkovsky,A.M., Xue,L., Fletcher,G.C., and Borutaite,V. (2002). Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? Biochimie. *84*, 233-240.

Tondera,D., Czauderna,F., Paulick,K., Schwarzer,R., Kaufmann,J., and Santel,A. (2005). The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells. J. Cell. Sci. *118*, 3049-3059.

Tondera,D., Grandemange,S., Jourdain,A., Karbowski,M., Mattenberger,Y., Herzig,S., Da Cruz,S., Clerc,P., Raschke,I., Merkwirth,C., Ehses,S., Krause,F., Chan,D.C., Alexander,C., Bauer,C., Youle,R., Langer,T., and Martinou,J.C. (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO. J. *28*, 1589-1600.

Tondera,D., Santel,A., Schwarzer,R., Dames,S., Giese,K., Klippel,A., and Kaufmann,J. (2004). Knockdown of MTP18, a novel phosphatidylinositol 3-kinase-dependent protein, affects mitochondrial morphology and induces apoptosis. J. Biol. Chem. *279*, 31544-31555.

Trushina,E., Dyer,R.B., Badger,J.D., Ure,D., Eide,L., Tran,D.D., Vrieze,B.T., Legendre-Guillemin,V., McPherson,P.S., Mandavilli,B.S., Van Houten,B., Zeitlin,S., McNiven,M., Aebersold,R., Hayden,M., Parisi,J.E., Seeberg,E., Dragatsis,I., Doyle,K., Bender,A., Chacko,C., and McMurray,C.T. (2004). Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. Mol. Cell. Biol. *24*, 8195-8209.

Tuominen,E.K., Wallace,C.J., and Kinnunen,P.K. (2002). Phospholipid-cytochrome c interaction: evidence for the extended lipid anchorage. J. Biol. Chem. *277*, 8822-8826.

Turmaine,M., Raza,A., Mahal,A., Mangiarini,L., Bates,G.P., and Davies,S.W. (2000). Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. *97*, 8093-8097.

Velier,J., Kim,M., Schwarz,C., Kim,T.W., Sapp,E., Chase,K., Aronin,N., and DiFiglia,M. (1998). Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. Exp. Neurol. *152*, 34-40.

Venkatraman,P., Wetzel,R., Tanaka,M., Nukina,N., and Goldberg,A.L. (2004). Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins. Mol. Cell. *14*, 95-104.

Verstreken,P., Ly,C.V., Venken,K.J., Koh,T.W., Zhou,Y., and Bellen,H.J. (2005). Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. Neuron. *47*, 365-378.

Vogel,F., Bornhovd,C., Neupert,W., and Reichert,A.S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. J. Cell. Biol. *175*, 237-247.

von Horsten,S., Schmitt,I., Nguyen,H.P., Holzmann,C., Schmidt,T., Walther,T., Bader,M., Pabst,R., Kobbe,P., Krotova,J., Stiller,D., Kask,A., Vaarmann,A., Rathke-Hartlieb,S., Schulz,J.B., Grasshoff,U., Bauer,I., Vieira-Saecker,A.M., Paul,M., Jones,L., Lindenberg,K.S., Landwehrmeyer,B., Bauer,A., Li,X.J., and Riess,O. (2003). Transgenic rat model of Huntington's disease. Hum. Mol. Genet. *12*, 617-624.

Vonsattel,J.P. and DiFiglia,M. (1998). Huntington disease. J. Neuropathol. Exp. Neurol. *57*, 369-384.

Vonsattel,J.P., Myers,R.H., Stevens,T.J., Ferrante,R.J., Bird,E.D., and Richardson,E.P., Jr. (1985). Neuropathological classification of Huntington's disease. J. Neuropathol. Exp. Neurol. *44*, 559-577.

Votruba,M., Moore,A.T., and Bhattacharya,S.S. (1997). Genetic refinement of dominant optic atrophy (OPA1) locus to within a 2 cM interval of chromosome 3q. J. Med. Genet. *34*, 117-121.

Votruba,M., Moore,A.T., and Bhattacharya,S.S. (1998). Clinical features, molecular genetics, and pathophysiology of dominant optic atrophy. J. Med. Genet. *35*, 793-800.

Wang,H., Lim,P.J., Karbowski,M., and Monteiro,M.J. (2009a). Effects of overexpression of huntingtin proteins on mitochondrial integrity. Hum. Mol. Genet. *18*, 737-752.

Wang,L., Dong,J., Cull,G., Fortune,B., and Cioffi,G.A. (2003). Varicosities of intraretinal ganglion cell axons in human and nonhuman primates. Invest. Ophthalmol. Vis. Sci. *44*, 2-9.

Wang,X. (2001). The expanding role of mitochondria in apoptosis. Genes Dev. *15*, 2922-2933.

Wang,X., Su,B., Lee,H.G., Li,X., Perry,G., Smith,M.A., and Zhu,X. (2009b). Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J. Neurosci. *29*, 9090-9103.

Wang,X., Zhu,S., Pei,Z., Drozda,M., Stavrovskaya,I.G., Del Signore,S.J., Cormier,K., Shimony,E.M., Wang,H., Ferrante,R.J., Kristal,B.S., and Friedlander,R.M. (2008). Inhibitors of cytochrome c release with therapeutic potential for Huntington's disease. J. Neurosci. *28*, 9473-9485.

Wanker,E.E., Rovira,C., Scherzinger,E., Hasenbank,R., Walter,S., Tait,D., Colicelli,J., and Lehrach,H. (1997). HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Genet. *6*, 487-495.

WARBURG,O. (1956). On respiratory impairment in cancer cells. Science. *124*, 269-270.

Wasiak,S., Zunino,R., and McBride,H.M. (2007). Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J. Cell. Biol. *177*, 439-450.

Waterham,H.R., Koster,J., van Roermund,C.W., Mooyer,P.A., Wanders,R.J., and Leonard,J.V. (2007). A lethal defect of mitochondrial and peroxisomal fission. N. Engl. J. Med. *356*, 1736-1741.

Welchman,R.L., Gordon,C., and Mayer,R.J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell. Biol. *6*, 599-609.

Wellington,C.L., Singaraja,R., Ellerby,L., Savill,J., Roy,S., Leavitt,B., Cattaneo,E., Hackam,A., Sharp,A., Thornberry,N., Nicholson,D.W., Bredesen,D.E., and Hayden,M.R. (2000). Inhibiting caspase cleavage of huntingtin reduces toxicity and

aggregate formation in neuronal and nonneuronal cells. J. Biol. Chem. *275*, 19831-19838.

Weydt,P., Pineda,V.V., Torrence,A.E., Libby,R.T., Satterfield,T.F., Lazarowski,E.R., Gilbert,M.L., Morton,G.J., Bammler,T.K., Strand,A.D., Cui,L., Beyer,R.P., Easley,C.N., Smith,A.C., Krainc,D., Luquet,S., Sweet,I.R., Schwartz,M.W., and La Spada,A.R. (2006). Thermoregulatory and metabolic defects in Huntington's disease transgenic mice implicate PGC-1alpha in Huntington's disease neurodegeneration. Cell. Metab. *4*, 349-362.

Wheeler,V.C., Auerbach,W., White,J.K., Srinidhi,J., Auerbach,A., Ryan,A., Duyao,M.P., Vrbanac,V., Weaver,M., Gusella,J.F., Joyner,A.L., and Macdonald,M.E. (1999). Length-dependent gametic CAG repeat instability in the Huntington's disease knock-in mouse. Hum. Mol. Genet. *8*, 115-122.

Wheeler,V.C., White,J.K., Gutekunst,C.A., Vrbanac,V., Weaver,M., Li,X.J., Li,S.H., Yi,H., Vonsattel,J.P., Gusella,J.F., Hersch,S., Auerbach,W., Joyner,A.L., and MacDonald,M.E. (2000). Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. Hum. Mol. Genet. *9*, 503-513.

White,J.K., Auerbach,W., Duyao,M.P., Vonsattel,J.P., Gusella,J.F., Joyner,A.L., and MacDonald,M.E. (1997a). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. Nat. Genet. *17*, 404-410.

White,J.K., Auerbach,W., Duyao,M.P., Vonsattel,J.P., Gusella,J.F., Joyner,A.L., and MacDonald,M.E. (1997b). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. Nat. Genet. *17*, 404-410.

Williams,A., Sarkar,S., Cuddon,P., Ttofi,E.K., Saiki,S., Siddiqi,F.H., Jahreiss,L., Fleming,A., Pask,D., Goldsmith,P., O'Kane,C.J., Floto,R.A., and Rubinsztein,D.C. (2008). Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat. Chem. Biol. *4*, 295-305.

Willis,S.N., Chen,L., Dewson,G., Wei,A., Naik,E., Fletcher,J.I., Adams,J.M., and Huang,D.C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes. Dev. *19*, 1294-1305.

Wong,E.D., Wagner,J.A., Gorsich,S.W., McCaffery,J.M., Shaw,J.M., and Nunnari,J. (2000). The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. J. Cell Biol. *151*, 341-352.

Wong,E.D., Wagner,J.A., Scott,S.V., Okreglak,V., Holewinske,T.J., Cassidy-Stone,A., and Nunnari,J. (2003). The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. J. Cell Biol. *160*, 303-311.

Wyttenbach,A., Carmichael,J., Swartz,J., Furlong,R.A., Narain,Y., Rankin,J., and Rubinsztein,D.C. (2000). Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. *97*, 2898-2903.

Wyttenbach,A., Swartz,J., Kita,H., Thykjaer,T., Carmichael,J., Bradley,J., Brown,R., Maxwell,M., Schapira,A., Orntoft,T.F., Kato,K., and Rubinsztein,D.C. (2001). Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington's disease. Hum. Mol. Genet. *10*, 1829-1845.

Xie,J., Marusich,M.F., Souda,P., Whitelegge,J., and Capaldi,R.A. (2007). The mitochondrial inner membrane protein mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11. FEBS. Lett. *581*, 3545-3549.

Yamaguchi,R., Lartigue,L., Perkins,G., Scott,R.T., Dixit,A., Kushnareva,Y., Kuwana,T., Ellisman,M.H., and Newmeyer,D.D. (2008). Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak oligomerization. Mol. Cell *31*, 557-569.

Yamamoto,A., Lucas,J.J., and Hen,R. (2000). Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell. *101*, 57-66.

Yang,Y., Gehrke,S., Imai,Y., Huang,Z., Ouyang,Y., Wang,J.W., Yang,L., Beal,M.F., Vogel,H., and Lu,B. (2006). Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc. Natl. Acad. Sci. U. S. A. *103*, 10793-10798.

Yang,Y., Ouyang,Y., Yang,L., Beal,M.F., McQuibban,A., Vogel,H., and Lu,B. (2008). Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. Proc. Natl. Acad. Sci. U. S. A. *105*, 7070-7075.

Yonashiro,R., Ishido,S., Kyo,S., Fukuda,T., Goto,E., Matsuki,Y., Ohmura-Hoshino,M., Sada,K., Hotta,H., Yamamura,H., Inatome,R., and Yanagi,S. (2006). A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO. J. *25*, 3618-3626.

Yoon,Y., Krueger,E.W., Oswald,B.J., and McNiven,M.A. (2003). The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol. Cell Biol. *23*, 5409-5420.

Youle,R.J. and Karbowski,M. (2005). Mitochondrial fission in apoptosis. Nat. Rev. Mol. Cell. Biol. *6*, 657-663.

Young,A.B., Greenamyre,J.T., Hollingsworth,Z., Albin,R., D'Amato,C., Shoulson,I., and Penney,J.B. (1988). NMDA receptor losses in putamen from patients with Huntington's disease. Science. *241*, 981-983.

Yu,Z.X., Li,S.H., Nguyen,H.P., and Li,X.J. (2002). Huntingtin inclusions do not deplete polyglutamine-containing transcription factors in HD mice. Hum. Mol. Genet. *11*, 905-914.

Zeevalk,G.D. and Nicklas,W.J. (1991). Mechanisms underlying initiation of excitotoxicity associated with metabolic inhibition. J. Pharmacol. Exp. Ther. *257*, 870-878.

Zeitlin,S., Liu,J.P., Chapman,D.L., Papaioannou,V.E., and Efstratiadis,A. (1995a). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. Nat. Genet. *11*, 155-163.

Zeitlin,S., Liu,J.P., Chapman,D.L., Papaioannou,V.E., and Efstratiadis,A. (1995b). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. Nat. Genet. *11*, 155-163.

Zeron,M.M., Fernandes,H.B., Krebs,C., Shehadeh,J., Wellington,C.L., Leavitt,B.R., Baimbridge,K.G., Hayden,M.R., and Raymond,L.A. (2004a). Potentiation of NMDA receptor-mediated excitotoxicity linked with intrinsic apoptotic pathway in YAC transgenic mouse model of Huntington's disease. Mol. Cell. Neurosci. *25*, 469-479.

Zeron,M.M., Fernandes,H.B., Krebs,C., Shehadeh,J., Wellington,C.L., Leavitt,B.R., Baimbridge,K.G., Hayden,M.R., and Raymond,L.A. (2004b). Potentiation of NMDA receptor-mediated excitotoxicity linked with intrinsic apoptotic pathway in YAC transgenic mouse model of Huntington's disease. Mol. Cell. Neurosci. *25*, 469-479.

Zeron,M.M., Hansson,O., Chen,N., Wellington,C.L., Leavitt,B.R., Brundin,P., Hayden,M.R., and Raymond,L.A. (2002). Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron. *33*, 849-860.

Zhou,H., Cao,F., Wang,Z., Yu,Z.X., Nguyen,H.P., Evans,J., Li,S.H., and Li,X.J. (2003). Huntingtin forms toxic NH2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity. J. Cell. Biol. *163*, 109-118.

Zoghbi,H.Y. and Orr,H.T. (2000). Glutamine repeats and neurodegeneration. Annu. Rev. Neurosci. *23*, 217-247.

Zou,H., Li,Y., Liu,X., and Wang,X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. *274*, 11549-11556.

Zuccato,C., Ciammola,A., Rigamonti,D., Leavitt,B.R., Goffredo,D., Conti,L., MacDonald,M.E., Friedlander,R.M., Silani,V., Hayden,M.R., Timmusk,T., Sipione,S., and Cattaneo,E. (2001a). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science. *293*, 493-498.

Zuccato,C., Ciammola,A., Rigamonti,D., Leavitt,B.R., Goffredo,D., Conti,L., MacDonald,M.E., Friedlander,R.M., Silani,V., Hayden,M.R., Timmusk,T., Sipione,S., and Cattaneo,E. (2001b). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science. *293*, 493-498.

Zuccato,C., Tartari,M., Crotti,A., Goffredo,D., Valenza,M., Conti,L., Cataudella,T., Leavitt,B.R., Hayden,M.R., Timmusk,T., Rigamonti,D., and Cattaneo,E. (2003). Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. Nat. Genet. *35*, 76-83.

Zuchner,S., Mersiyanova,I.V., Muglia,M., Bissar-Tadmouri,N., Rochelle,J., Dadali,E.L., Zappia,M., Nelis,E., Patitucci,A., Senderek,J., Parman,Y., Evgrafov,O., Jonghe,P.D., Takahashi,Y., Tsuji,S., Pericak-Vance,M.A., Quattrone,A., Battologlu,E.,

Polyakov,A.V., Timmerman,V., Schroder,J.M., and Vance,J.M. (2004). Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat. Genet. *36*, 449-451.

Zuchner,S. and Vance,J.M. (2006). Mechanisms of disease: a molecular genetic update on hereditary axonal neuropathies. Nat. Clin. Pract. Neurol. *2*, 45-53.

Zunino,R., Braschi,E., Xu,L., and McBride,H.M. (2009). Translocation of SenP5 from the nucleoli to the mitochondria modulates DRP1-dependent fission during mitosis. J. Biol. Chem. *284*, 17783-17795.

Zunino,R., Schauss,A., Rippstein,P., Andrade-Navarro,M., and McBride,H.M. (2007). The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. J. Cell. Sci. *120*, 1178-1188.