1 INTRODUCTION

Mitochondria are essential mammalian organelles surrounded by two lipid bilayers (Alberts et al., 1995). This complex structural feature is mirrored by their "all-round" participation in life and death of the cell. Mitochondria are highly dynamic and due to their two membranes, fusion and fission events are extremely complex. Mitochondrial diseases are heterogeneous group of disorders, often characterized by morphological changes in the mitochondria, a defective respiratory chain and variable symptoms, ranging from severe metabolic disorders with onset in early infancy or childhood to late onset adult myopathies. Mutations in mitochondrial DNA are the most frequent cause of mitochondrial diseases in adults. However, the mtDNA encodes only a subset of proteins of the different complexes of the respiratory chain. Nuclear genes encode all the other mitochondrial proteins and most of the mitochondrial disorders are caused by mutations in the nuclear genes. The symptoms of these diseases vary depending on which mitochondrial functions are disturbed, and not all of them cause morphological changes in the mitochondria. In general terms, tissues and organs (retina, optic nerve, brain, heart, testis, muscle, etc.) that are heavily dependent upon oxidative phosphorylation bear the brunt of the pathology. It is also puzzling that many mitochondrial disorders affect multiple organ systems, whereas others have a highly stereotyped and organ specific phenotype. These subtle interactions between nuclear and mitochondrial genes in health and disease will have broader relevance for our understanding of many inherited and sporadic neurological disorders.

1.1 Mitochondria – structure and function

Mitochondria are small cytoplasmic organelles remnant of a prokaryotic organism that had become a vital symbiotic partner to the eukaryotic cell early in the evolution (Gray et al., 1999). Mitochondria contain their own DNA, which is maternally inherited. Mitochondria adopt different shapes depending on the cell type and the metabolic demands of the cell. Their two predominant morphologies are reflected in the Greek name of the organelle – 'mitos' for thread and 'chondros' for grain. Mitochondria are usually 0.5-1 µm in size and are bounded by two membranes. The smooth outer membrane contains many copies of a transport protein porin, which forms aqueous channels allowing molecules with a maximal molecular weight of 5000 Daltons to penetrate the membrane. The inner membrane is conspicuously folded, forming tubular or lamellar structures called cristae, which are connected to it by narrow tubular structures, cristae junctions (Fig. 1.1). The core of the mitochondria is the matrix that harbours the mtDNA.

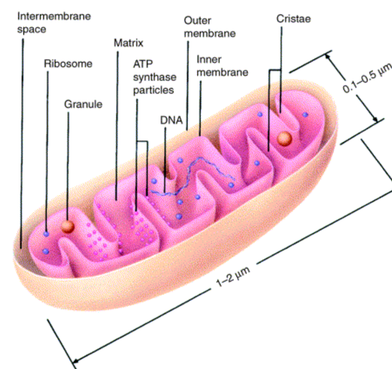

Figure 1.1: A schematic image of a mitochondrion.
(Frey and Mannella, 2000).

Two distinct genetic systems encode mitochondrial proteins: mtDNA and nuclear DNA (nDNA). mtDNA is a small 16.6 kb circle of double stranded DNA that codes for 13 respiratory chain polypeptides of respiratory complexes I, III, IV, and V (only complex II is solely composed of proteins encoded by nuclear genes) and 24 nucleic acids necessary for intra-mitochondrial protein synthesis (Anderson et al., 1981). Mitochondria are intimately involved in cellular homeostasis. They nourish cells by converting energy from carbon sources into ATP (adenosine triphosphate). Additionally, they are important for thermeogenesis, calcium and iron homeostasis, intracellular signalling and apoptosis, various metabolic intermediary pathways including metabolism of amino acids, lipids, cholesterol, steroids and nucleotides through oxidative phosphorylation (OXPHOS).

Oxidative metabolism is fuelled by pyruvate generated from carbohydrates in glycolysis and fatty acids produced from triglycerides. These are selectively imported into the mitochondrial matrix and broken down into acetyl CoA by the pyruvate dehydrogenase complex or the β-oxidation pathway. The acetyl group then participates in the citric acid cycle, which produces substrates for OXPHOS like NADH and FADH. Electrons generated from NADH are passed along a series of carrier molecules called the electron transport chain (ETC), the products of this process being water and ATP. In this process, protons are pumped from the matrix across the mitochondrial inner membrane through respiratory complexes: I, III, and IV. When protons return to the mitochondrial matrix down their electrochemical gradient, ATP is synthesized via complex V (ATP synthase). Thus the mitochondrion converts energy derived from chemical fuels by an OXPHOS process that is more efficient than anaerobic glycolysis. In the mitochondrion the metabolism of one molecule of glucose produces about 30 molecules of ATP, while only two molecules of ATP are produced by glycolysis in the cytoplasm. This means that organs with a high-energy demand are vulnerable to the depletion of mitochondrial energy production.

Mitochondria depend a lot on nDNA since nuclear genes code for the majority of mitochondrial respiratory chain polypeptides (Shoubridge, 2001). These polypeptides are synthesised in the cytoplasm with a mitochondrial targeting sequence that directs them through the translocation machinery spanning the outer and inner membranes. The targeting sequence is then cleaved before the subunit is assembled with its counterparts on the inner mitochondrial membrane. The components of the import machinery ("*TIM*" and "*TOM*" proteins) and the respiratory chain assembly proteins are all the products of nuclear genes. Nuclear genes are also important for maintaining the mitochondrial genome, including those encoding the mitochondrial DNA polymerase γ (*POLG1*) (Van Goethem et al., 2001) and products that maintain an appropriate level of free nucleotides within the mitochondrion (Nishino et al., 1999; Saada et al., 2001; Mandel et al., 2001; Spelbrink et al., 2001). nDNA also codes for essential factors needed for intra-mitochondrial transcription and translation, including *TFAM*, *TFBM1* and *TFBM2* (Larsson et al., 1998; Falkenberg et al., 2002). A disruption of both nuclear and mitochondrial genes can therefore cause mitochondrial dysfunction leading to human disease (Chapter 7.1).

1.2 Mitochondrial Dynamics

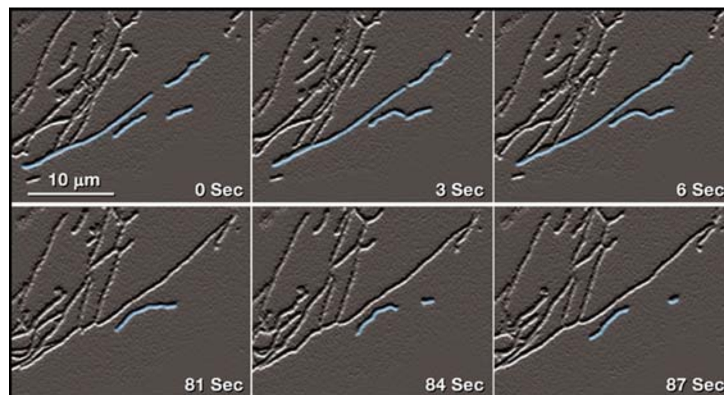

Figure 1.2: Mitochondrial Fusion and Fission.
Still frames from time-lapse fluorescence movie of YFP-labelled mitochondria in living mouse embryonic fibroblasts. Selected mitochondria are highlighted. In the first three frames, two pairs of mitochondria contact end-to-end and immediately fuse. In the last three frames, the lower fusion product undergoes fission, and the daughter mitochondria move apart (Chen et al., 2003).

Any given mitochondrion is not a discrete, autonomous organelle. In fact, the identity of an individual mitochondrion is short-lived, because it will fuse with a neighbouring mitochondrion in the near future. Therefore, the entire mitochondrial population is in constant flux, driven by continual fusion and division of mitochondria (Fig. 1.2), with the relative rates of these two processes dictating the average size and degree of connectivity of the mitochondrial 'network'. If two or more cultured mammalian cells are artificially fused to

form a cell hybrid, the mitochondrial populations are completely fused within 8 hr (Chen et al., 2003; Legros et al., 2002; Mattenberger et al., 2003).

Table 1.1: Mitochondrial shaping proteins in mammals and yeast

Human proteins	*S. cerevisiae* homologs	Location	Function
OPA1	Mgm1	IMS, IM/OM, peripheral/integrated	Fusion
Mfn1/2	Fzo1	OM integrated	Fusion
	Ugo1	OM integrated	Fusion
DRP1/DLP1	Dnm1	Cytosol, OM peripheral	Fission
hFis	Fis	OM integrated	Fission
	Mdv1	Cytosol, OM peripheral	Fission
	Caf4	Cytosol, OM peripheral	Fission
MTP18		IMS, mitochondrial membranes integrated	Fission
	Mdm33	IM integrated	IM fission?
	Mdm10	OM integrated	Tubulation
	Mdm12	OM integrated	Tubulation
	Mdm31	IM integrated	Tubulation
	Mdm32	IM integrated	Tubulation
	Mmm1	OM/IM-spanning	Tubulation
	Mmm2	OM integrated	Tubulation
Endophilin B1		Cytosol, dynamic association with mitochondria	OM remodelling
Mitofilin	YKR016W	IMS	Cristae remodelling
ATP synthase	ATP synthase	IM, F0-integrated/ F1-peripheral	Cristae remodelling

IMS- inter membrane space; IM- inner membrane; OM-outer membrane

1.2.1 The fusion machinery

There are several important characteristics of mitochondria that 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 mitochondrial fusion reaction (Nunnari et al., 1997). Indeed, the three known mitochondrial fusion molecules appear solely dedicated to mitochondrial fusion, suggesting the machinery evolved independently and is uniquely tailored for this organelle. Second, mitochondria have an outer and inner membrane, with a membrane potential across the latter. 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. This implies symmetry in the distribution of molecules between the adjacent membranes and is undoubtedly reflected in their fusion mechanism. In spite of these unique features, mitochondrial fusion likely has some general features in common with virus-mediated and SNARE-mediated membrane fusion. The specificity of membrane fusion is likely determined by the formation of specific protein complexes formed *in-trans* between the fusing membranes. Moreover, conformational changes *in-trans* protein complexes will likely provide the energy necessary to oppose two negatively charged lipid bilayers.

OPA1 plays an important role in the control of inner membrane structure through fusion (Chen et al, 2005; Cipolat et al, 2004). Knockdown of OPA1 by RNA interference (RNAi) leads to mitochondrial fragmentation that is due to loss of mitochondrial fusion (Chen et al.

2005; Cipolat et al. 2004; Griparic et al. 2004). In addition to loss of fusion, OPA1 RNAi also leads to severe aberrations in cristae structure (Griparic et al. 2004; Olichon et al. 2003). Explaining OPA1 function is complicated by the fact that OPA1 overexpression can also lead to mitochondrial fragmentation or elongation, depending on the experimental system (Chen et al. 2005; Cipolat et al. 2004; Griparic et al. 2004; Olichon et al. 2003).

The mitofusins, Mfn1 and Mfn2, are the only mammalian outer membrane GTPases known to be essential for mitochondrial fusion (Chen et al., 2003; Rojo et al., 2002; Santel and Fuller, 2001). Mitofusins are required on adjacent mitochondria during fusion and form complexes *in-trans* that tether mitochondria together (Koshiba et al., 2004). Deletion of either Mfn1 or Mfn2 results in mitochondrial fragmentation and poor mitochondrial function (Chen et al., 2005), although low levels of mitochondrial fusion remain. Moreover, Mfn1 and Mfn2 can functionally replace each other and deletion of both mitofusins abolishes all mitochondrial fusion (Koshiba et al, 2004; Chen et al, 2005). In spite of the functional similarities of Mfn1 and Mfn2, differences for these molecules in relation to OPA1 have been described. OPA1 function shows dependence on Mfn1 but not on Mfn2 (Cipolat et al., 2004), and Mfn1 displays more mitochondrial tethering activity (Ishihara et al., 2004).

Mitochondrial fusion is well documented in yeast. Mgm1p (ortholog of OPA1), Fzo1p (ortholog of Mfn1/2) and Ugo1p are the three major components involved in the yeast mitochondrial fusion pathway. Mgm1Δ cells display no mitochondrial fusion, even when the mitochondria are in close contact, indicating that Mgm1p, like Fzo1p, is required at a late step in the fusion pathway (Sesaki et al., 2003a; Wong et al., 2003). In addition, mgm1Δ yeast have dramatically swollen and poorly involuted cristae (Sesaki et al., 2003a). However, the cristae defects in mgm1Δ cells are largely suppressed in dnm1Δ/mgm1Δ cells, but these cells nevertheless are completely defective for mitochondrial fusion (Sesaki et al., 2003a; Wong et al., 2003). Correct processing of Mgm1p by Rbd1p/Pcp1p is critical to its function in mitochondrial dynamics. Rbd1/Pcp1Δ cells produce only l-Mgm1p (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003b), which display severely fragmented mitochondrial morphology, indicating l-Mgm1p is not sufficient to support normal levels of mitochondrial fusion. s-Mgm1p targeted to mitochondria by fusion of a heterologous N-terminal mitochondrial targeting signal fails to complement the fusion defect in mgm1Δ cells also (Herlan et al., 2003). The ratio of the two isoforms is critical for fusion because mutations that cause differences in the ratio of the two isoforms cause defects in mitochondrial morphology (Herlan et al., 2003 and 2004; McQuibban et al., 2003; Sesaki et al., 2003b). Although OPA1/Mgm1 protein is not exposed to the cytosol, cells lacking OPA1

or yeast lacking Mgm1 have no detectable outer membrane fusion (Chen et al., 2005, Sesaki et al., 2003b).

A third component of the fusion pathway is Ugo1p, embedded in the mitochondrial outer membrane of yeast (Sesaki et al., 2001). However, no Ugo1p homologs have been identified outside of the fungi kingdom, suggesting that its role in mitochondrial fusion is not conserved. Ugo1p, Fzo1p and Mgm1p assemble into a fusion complex. The N-terminal cytoplasmic domain of Ugo1p binds Fzo1p near its transmembrane region and the C-terminal half of Ugo1p binds Mgm1p in the inter-membrane space (Sesaki et al., 2004). Interestingly, the GTPase activities of Fzo1p and Mgm1p are not required for binding to Ugo1p, suggesting that Ugo1p binding does not require the GTP hydrolysis by either protein (Sesaki et al., 2001). Thus Ugo1p provides a scaffold for the assembly of a fusion complex that spans the outer and inner membranes, and provides a link that may coordinate their fusion.

Coordination of outer and inner membrane fusion

The double membrane architecture of mitochondria means that fusion necessarily entails merging four membranes into two membranes. It is commonly assumed that outer and inner membrane fusions are tightly coordinated. Manipulation of mitochondrial fusion *in vitro*, however, has distinguished outer membrane and inner membrane fusion as two mechanistically distinct processes (Meeusen et al., 2004). Under limiting GTP concentrations *in vitro*, mitochondria will fuse their outer membranes, but not inner membranes. Supplementation with a GTP regenerating system allows for subsequent inner membrane fusion. Dissipation of mitochondrial membrane potential has been reported to block mitochondrial fusion. In cultured mammalian cells, mitochondrial fusion is disrupted by H^+ or K^+ ionophores that perturb the mitochondrial membrane potential (Ishihara et al., 2003; Legros et al., 2002; Mattenberger et al., 2003). Upon closer examination, it was found that these ionophores allow outer but not inner membrane fusion (Malka et al., 2005). Remarkably, such uncoupling can also be observed in untreated cells, suggesting that inner and outer membrane fusion may not be as tightly coordinated as commonly is assumed (Malka et al., 2005).

1.2.2 The fission machinery

Drp1 is a key component of the mitochondrial fission machinery. Much of Drp1 is located in the cytosol, but a subpool is localized to punctate spots on mitochondrial tubules, and a subset of these spots mark future sites of fission (Smirnova et al., 2001). Inhibition of Drp1 by expression of a dominant-negative mutant or by RNAi leads, owing to inhibition of fission, to

elongation of mitochondrial tubules (Lee et al., 2004; Smirnova et al., 2001). Like dynamin, the yeast Drp1 ortholog, Dnm1, also assembles into multimeric complexes that constricts lipid membranes (Ingerman et al., 2005). Therefore Drp1 plays a role in membrane constriction during the process of mitochondrial fission. Fis1 is another component required for mitochondrial fission, which is localised uniformly on the mitochondrial outer membrane. Overexpression of Fis1 leads to mitochondrial fragmentation that is dependent on Drp1 (James et al., 2003; Yoon et al., 2003). Knockdown of Fis1 causes elongation of mitochondrial tubules, much as with inhibition of Drp1 (Lee et al., 2004). Surprisingly, knockdown of Fis1 in mammalian cells by RNA interference does not disrupt the mitochondrial localization of Drp1 (Lee et al., 2004).

The mechanisms recruiting fission complexes to mitochondria are best understood in yeast, where Dnm1 localization to mitochondria clearly relies on the outer mitochondrial protein Fis1 and two adaptor proteins (Mdv1 and Caf4) (Griffin et al., 2005; Mozdy et al., 2000; Okamoto and Shaw, 2005; Tieu et al., 2002). Fis1 works through Mdv1 and a related protein, Caf4, to recruit Dnm1 to punctate spots on mitochondria (Griffin et al., 2005). Mdv1 and Caf4 both physically interact with Fis1 and Dnm1, thereby allowing them to act as molecular adaptors in the assembly of the fission complex. In the absence of Mdv1 and Caf4, it is unclear how the mammalian fission machinery is assembled on the mitochondrial surface.

Several other molecules like Endophilin B1, MTP18, GDAP, Miro-1 and Miro-2 have been proposed to regulate mitochondrial dynamics in mammals (Karbowski et al., 2004a; Tondera et al., 2005; Niemann et al., 2005; Farnsson et al., 2006)

1.3 Mitochondrial dynamics and apoptosis

Several studies have indicated that mitochondrial morphology changes during apoptosis, resulting in small, round and more numerous organelles (Mancini et al., 1997; Desagher and Martinou, 2000; Frank et al., 2001; Pinton et al., 2001; Jagasia et al., 2005). This process of fragmentation occurs early in the cell death pathway around the time that pro-apoptotic members of the Bcl2 family, Bax and Bak, translocate from the cytosol to mitochondria. This relocalisation occurs before caspase activation and coalesces on the mitochondrial surface into large foci. This leads to outer membrane permeabilisation, which allows cytochrome c sequestered in the mitochondrial intermembrane space to be released into the cytosol (Frank et al., 2001; Capano and Crompton, 2002; Karbowski et al., 2004b). Mitochondrial fragmentation during apoptosis is dependent on the normal mitochondrial fission machinery, and inhibition of Drp1 or Fis1 prevents mitochondrial fragmentation, inhibits cytochrome c

release, and can delay or reduce the extent of cell death (Breckenridge et al., 2003; Frank et al., 2001; Lee et al., 2004). In addition, ectopic expression of wild type Drp1 induces mitochondrial fragmentation and cell death. Cuddeback and co-workers (2001) reported that Endophilin B1 could bind to Bax upon induction of apoptosis. Elevating the levels of Endophilin B1 increases cell sensitivity to apoptosis, which is consistent with a role for this protein in cell death (Cuddeback et al., 2001; Pierrat et al., 2001).

In contrast, mitochondrial fusion plays a protective role in apoptosis. Inhibition of mitochondrial fusion facilitates cell death in response to some apoptotic signals (Olichon et al., 2003; Sugioka et al., 2004). Mitochondrial fusion is reduced following induction of apoptosis (Karbowski et al. 2004b) and overexpression of mitofusins can reduce the level of apoptosis (Sugioka et al., 2004). Depletion of OPA1 or mitofusins results in poor cell growth (Chen et al., 2005) and enhances susceptibility to apoptotic stimuli (Lee et al., 2004; Olichon et al., 2003; Sugioka et al., 2004). In mammals, cytochrome c release from the mitochondria is a key event leading to activation of caspases. The cristae of mitochondria rearrange their morphology and open up at their region of contact with the outer membrane and /or become wider on exposure to BH3-only members of Bcl2 family, Bid and Bik, and this has been suggested to stimulate the release of cytochrome c. Recent studies revealed cristae remodelling during apoptosis, which leads to the release of cytochrome c, is regulated by OPA1 and PARL (Cipolat et al., 2006; Frezza et al., 2006).

1.4 Mitochondria and neurodegenerative diseases

Different cells and tissues have distinct sensitivities and responses to mitochondrial dysfunction. These differences are probably due to the cell-type specializations that rely on particular functions of mitochondria.

Table 1.2: Mitochondrial proteins mutated in neurodegenerative diseases

Disease	Gene	Function
Charcot-Marie-Tooth type 2A	Mfn2	Mitochondrial fusion
Charcot-Marie-Tooth type 4A	GDAP1	Mitochondrial dynamics/fission?
Dominant optic atrophy	OPA1	Mitochondrial fusion and cristae structure
Recessive optic atrophy	OPA3	Unknown
Leber's hereditary optic neuropathy	ND genes in mtDNA	Oxidative phosphorylation
Hereditary spastic paraplegia	Paraplegin	Mitochondrial ATPase/protease
Hereditary spastic paraplegia	HSP60	Mitochondrial heat shock protein
Familial amyotrophic lateral sclerosis	SOD1	Superoxide dismutase
Familial Parkinson's disease	PINK1	Mitochondrial serine/threonine kinase
Friedreich's ataxia	Frataxin	Formation of iron-sulphur clusters in mitochondria

Neurons appear particularly vulnerable to mitochondrial dysfunction, and many diseases of mitochondria result in neurodegeneration. In part, this vulnerability may be due to their extreme physical dimensions and the need to actively recruit mitochondria to nerve terminals. Classic mitochondrial encephalomyopathies caused by mtDNA mutations are often characterized by neurological symptoms (Dimauro and Davidzon, 2005; Taylor and Turnbull, 2005). Prominent are those associated with movement, such as ataxia, dysarthria and peripheral neuropathy. Mutations in mitochondrial proteins that are encoded in the nucleus have also been linked to neurodegenerative diseases (Beal, 2005).

1.4.1 Autosomal dominant optic atrophy

Autosomal dominant optic atrophy (ADOA-MIM #165500) also known as Kjer's optic atrophy, is the most common form of inherited optic neuropathy, with a frequency of 1:12,000 to 1:50,000 (Kjer et al., 1996). The gene responsible for ADOA mapping to human chromosome 3q28 (Eiberg et al., 1994) was identified (Alexander et al., 2000; Delettre et al., 2000) and designated Optic Atrophy type 1 (OPA1). The cardinal sign of the disease is a symmetric optic atrophy visible in the fundus as a temporal pallor of the optic disc (Fig. 1.3), meaning that some fibres from the central retina (macula) have been lost, in contrast to those of the peripheral retina that are retained. In aged patients or in severe cases, the optic disc may appear diffusely atrophic. This disease is also characterized by progressive loss of visual acuity, bilateral atrophy of the optic nerve, central visual field defects and colour vision deficits (Kline and Glaser, 1979; Votruba et al., 1998).

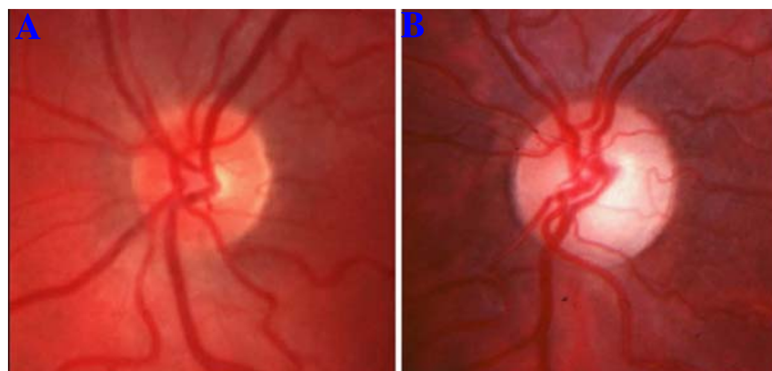

Figure 1.3: Eye fundus photographs.
A) Fundus of normal individual, B) showing that the patient's optic nerve disc is pale and that pallor predominates in the temporal side (Olichon et al., 2006).

ADOA is usually discovered at around 3-6 years of age, but depending on the level of visual loss, this may be earlier as in cases with nystagmus detected as young as 1 year of age (Kline and Glaser, 1979), or later. The presence of optic atrophy in young patients suggests that the disease is congenital. There is considerable variation in the visual function among patients,

even within the same family (Votruba et al, 1998). The disease is highly variable in severity, exhibiting inter and intrafamilial variations ranging from asymptomatic carriers to legally blind patients (Votruba et al., 1998).

Electrophysiological studies have suggested that the underlying defect is a retinal ganglion cell (RGC) degeneration leading to atrophy of the optic nerve (Johnston et al., 1979; Kjer et al., 1983), as observed in Leber's hereditary optic neuropathy (Carelli et al., 2004) and this is followed by ascending optic atrophy. Histopathological studies have demonstrated a decrease in RGC amount with gliosis of the ganglion cell layer that predominates in central retina. In addition, there is loss in myelin sheaths and neurofibrils within the optic nerve, optic chiasm, and optic tracts (Johnston et al., 1979; Kjer et al., 1983). Reduced and delayed amplitudes of the pattern visual-evoked potentials and reduced amplitude of the ganglion cell-specific N95 component of the pattern electroretinogram are consistent with this finding (Smith, 1972; Elenius et al., 1991). Intriguingly, metabolic studies of ADOA patients suggest a deficit in mitochondrial function within the skeletal muscle (Lodi et al., 2004). Delettre and co-workers (2000) reported that monocytes from ADOA patients have aggregated mitochondria, suggesting a defect in mitochondrial distribution within cells. These patients also show lower rates of ATP synthesis in skeletal muscle and fewer copies of mtDNA (Kim et al., 2005). Recently, it became clear that this disease might have additional parallels to classic mitochondrial encephalomyopathy. In some pedigrees of ADOA caused by OPA1 mutations, there is associated neurosensory hearing loss, ptosis and ophthalmoplegia (Payne et al., 2004).

1.4.2 Leber's hereditary optic atrophy

Leber's hereditary optic neuropathy (LHON) is transmitted by non-Mendelian, mitochondrial inheritance and is due to mutations in the mitochondrial DNA. As mitochondria are maternally inherited (Giles et al., 1980) there is no male-to-male transmission in an LHON pedigree. It is characterized by acute bilateral vision loss and colour vision deficits (Newman et al., 1991; Nikoskelainen et al., 1996). Histopathology from LHON patients with long-standing visual loss shows axonal degeneration in the optic nerve and loss of myelin, and there is evidence that the small axons of the papillomacular bundle, found centrally in the optic nerve, are particularly vulnerable (Carelli et al., 2002; Sadun et al., 2000). The respiratory dysfunction may lead to axoplasmic stasis and swelling, with evidence of demyelination. This may be reversible, as evidenced by the possibility of recovery of vision, but if the mitochondrial apoptotic pathway is activated, as it is most likely (Howell, 1997), ganglion cells are permanently lost.

1.4.3 Charcot-Marie-Tooth disease

CMT (Charcot-Marie-Tooth) is a group of diseases characterized by motor defects in at least the lower extremities (Zuchner and Vance, 2005). The most common form of CMT (CMT1) involves defects in the Schwann cells (Kuhlenbaumer et al. 2002; Zuchner and Vance, 2005), which result in reduced nerve conduction velocities and show demyelination. Another axonal type of CMT (CMT2A) with associated optic atrophy has also been linked to mutations in Mfn2 (Zuchner et al., 2006). In contrast to CMT1, patients with CMT2A have normal nerve conduction velocities, and the peripheral nerve defect is thought to be axonal or intrinsic to the motor and sensory neurons. CMT4A - a demyelinating, recessive form of CMT - is caused by mutations in GDAP1 expressed in both neurons and Schwann cells (Baxter et al., 2002). CMT4A patients can have features of myelin or axonal defects, or a combination of both. These strongly emphasize the sensitivity of long peripheral nerves to perturbations in mitochondrial dynamics.

1.5 OPA1

1.5.1 Gene structure and expression

The OPA1 gene is 6031 nucleotides long and is composed of 31 exons (Delettre et al., 2001) spanning a region of 60-90 kb of genomic DNA. It encodes a mitochondrial dynamin-related GTPase protein of 960 amino acids. Northern blot analyses of OPA1 transcripts have shown the presence of OPA1 RNA in almost all human tissues examined (Alexander et al., 2000).

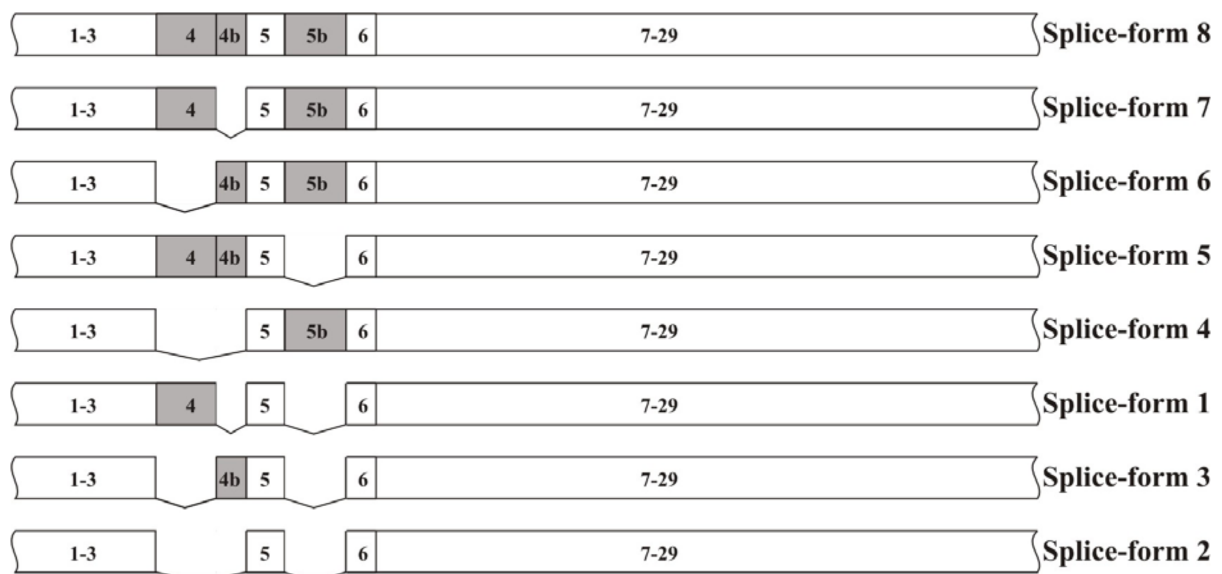

Figure 1.4: Splice variants of human OPA1.
Alternative splicing involving exons 4, 4b and 5b (shaded regions) in OPA1 generate eight splice-forms.

The last exon is not coded, and exons 4, 4b, and 5b generate eight splice forms of RNA by alternative splicing (Fig. 1.4), with some splicing forms predominating in the brain, retina, heart and muscle (Delettre et al., 2001).

1.5.2 Protein structure and localisation

The OPA1 is a member of the dynamin-related GTPase protein (DRP) subfamily. DRPs and classical dynamins all encode GTPase, middle, and GTPase effector domains (GED) (Fig. 1.5a). Unlike dynamins, DRPs lack a pleckstrin homology (PH) domain and a proline-rich domain (PRD) (Praefcke and McMahon, 2004). OPA1 shares a number of structural features with proteins of the dynamin family (Fig. 1.5b) and functionally is closely related to a family of proteins involved in the mitochondrial network organisation. These include a GTPase domain containing the three consensus GTP binding sequences (G1, G3 and G4) and the dynamin signature (G2), a middle domain and a C-terminal coiled-coil region (CC-II), which may correspond to a GTPase effector domain (GED).

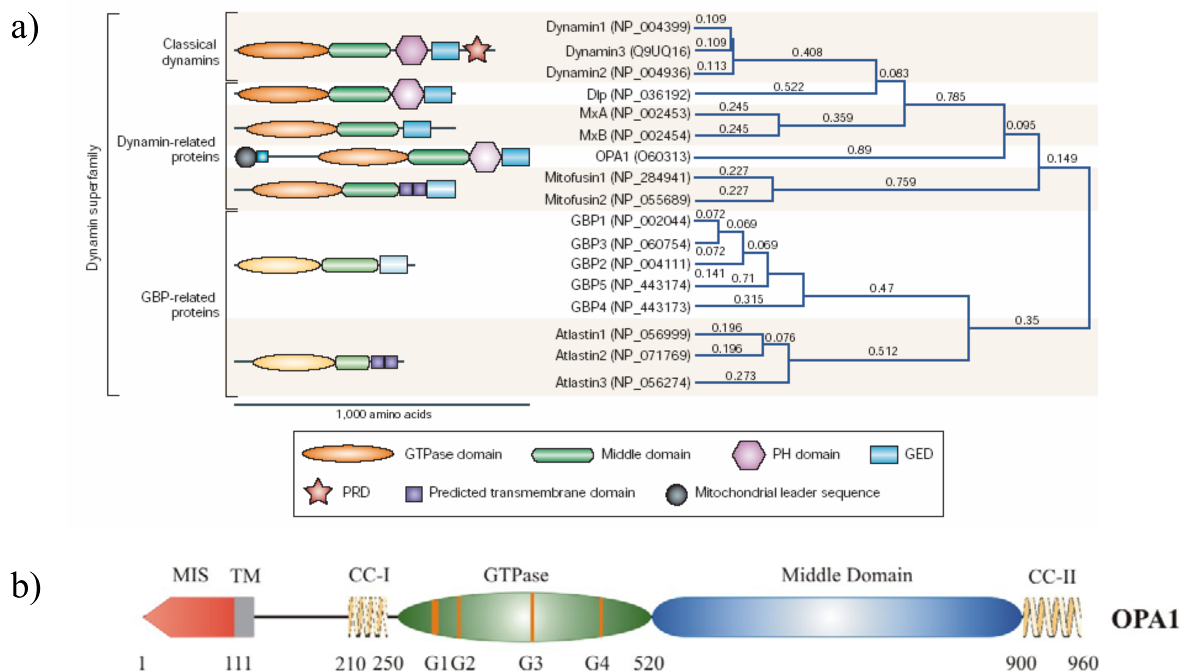

Figure 1.5: Domain structure of the human dynamin super family.

a) Domains are represented by different symbols and the decreasing intensities of the colours correspond to decreasing sequence homology. All dynamins contain a GTPase domain that binds and hydrolyses GTP, a middle domain and a GTPase effector domain (GED) that are involved in oligomerisation and stimulation of GTPase activity. Additionally, most dynamins contain a domain for interactions with lipid membranes. This can be a pleckstrin-homology (PH) domain, a transmembrane domain or a sequence for lipid attachment. Classical dynamins contain a proline-rich domain (PRD) at the carboxyl terminus that interacts with Src-homology-3 (SH3) domains. Human dynamin super family members have been grouped according to their domain structure and their accession numbers are shown. The family tree was calculated in Mac Vector using the tree building method with Poisson-corrected distances. DLP1, dynamin-like protein 1; GBP1, guanylate-binding protein 1; OPA1, optic atrophy 1 (Praefcke and McMahon, 2004).

b) Schematic representation of OPA1 protein isoform 1 domain structure.

The G1 motif (in the so-called P-loop) coordinates the phosphates, whereas the threonine in the G2 motif is involved in catalysis. The glycine in the G3 motif forms a hydrogen bond with the γ-phosphate of GTP. The G4 motif is involved in base and ribose coordination. The functional importance of the middle domain is unknown. The carboxy terminus of OPA1 differs from other dynamin family members in lacking a PRD as well as dynamin GTPase effector and PH domains (Delettre et al., 2002). In dynamins PH domain binds to lipids, while the association of OPA1 with mitochondrial membrane is due to the presence of transmembrane domain (TM) located immediately after the highly basic amino-terminal mitochondrial import sequence (MIS). Primary structure predictions suggest a high probability of coiled coil formations in two separate domains encoded by exons 6-7 (100%) and exons 27-28 (95%). OPA1 shows 33% homology to Mgm1 and 31% homology to Msp1, both members of a subfamily of dynamins found in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* where they have been shown to play a role in the maintenance of the mitochondrial genome and mitochondrial morphology.

Immunofluorescence and biochemical evidence suggests that the highly basic amino terminal extension is essential for OPA1 targeting to mitochondria (Misaka et al., 2002; Olichon et al., 2002; Satoh et al., 2003). OPA1 is an intermembrane space protein, closely associated with the inner membrane (Olichon et al., 2002). The OPA1 gene produces multiple protein isoforms, owing to extensive alternative splicing that yields eight mRNA isoforms (Delettre et al., 2001).

1.6 Is haploinsufficiency the only cause of type 1-ADOA?

To date 117 OPA1 gene mutations, mainly of familial origin, have been described so far (http://lbbma.univ-angers.fr/eOPA1/) (Ferre et al., 2005). The OPA1 mutations are spread throughout the coding sequence of the gene, but most (90%) are distributed within exons 8 to 28 with a majority localised in the GTPase domain, whereas the 5' region is little affected (Fig. 1.6). No obvious correlation between genotype and phenotype could be defined (Puomila et al., 2005; Thiselton et al., 2002; Pesch et al., 2001). Two of the numerous different OPA1 mutations, one corresponding to a deletion of the entire gene and the other abolishing the entire ORF (Trp2Stop), provide evidence that haploinsufficiency is the cause of the disease (Pesch et al., 2001; Marchbank et al., 2002). Accordingly, almost 50% of the OPA1 mutations cause premature truncations of OPA1. Furthermore, the evaluation of mutation spectrum suggests more than one pathogenetic mechanism for the disease.

GTPase activity has indeed been shown to be necessary for the function of Mgm1p/OPA1 in mitochondrial fusion (Wong et al., 2003; Sesaki et al., 2003a; Griparic et al., 2004; Cipolat et al., 2004). Nevertheless, many researchers have demonstrated that expression of GTPase mutants of Mgm1p and Msp1p in yeast (and OPA1 in MEFs) containing a wild type allele of the appropriate dynamin induces mitochondrial fragmentation by a dominant negative effect (Wong et al., 2003; Guillou et al., 2005; Shepard and Yaffe, 1999; Cipolat et al., 2004).

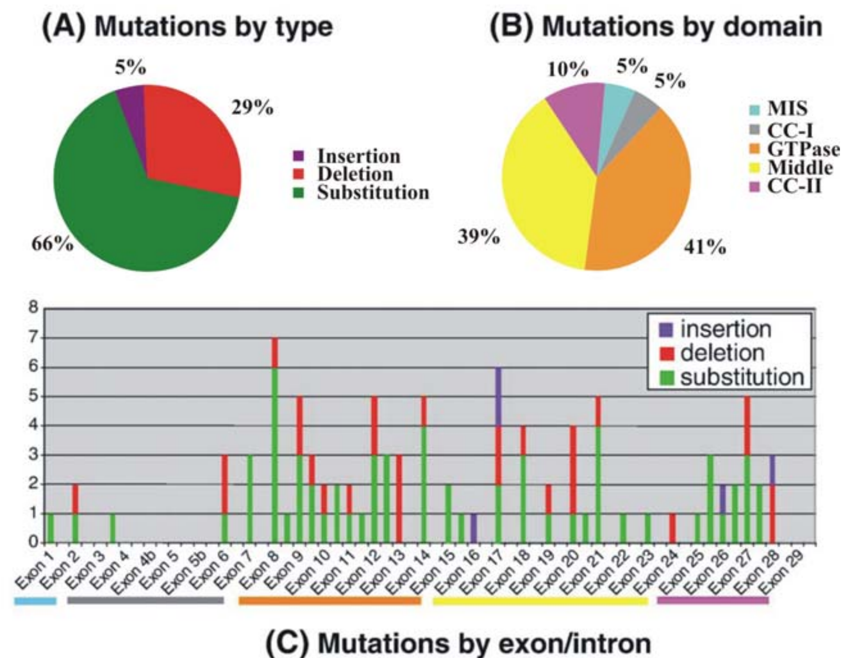

Figure 1.6: Mutation spectrum of OPA1.
Distribution of the mutations of OPA1 gene according to their type (A), domain (B) and localization (C). In B, only mutations affecting exons are considered and domains correspond to those described in Fig. 1.5b. In C, spaces between two consecutive exons correspond to introns and colored bars under exons indicate their belonging to OPA1 domains. (Olichon et al., 2006)

The integration of OPA1 GTPase mutants into protein complexes together with wild type OPA1 may abolish the activity of the dynamin and thus the fusion competence of mitochondria. However, while oligomerisation of Mgm1p has been deduced using genetic approaches (Wong et al., 2003), no data concerning the ability of OPA1 to oligomerise, and the effect of oligomerisation on its GTPase activities are available. Mutations occurring in the GTPase domain may exert a dominant negative effect, while carboxy terminal truncations, by removing the potential GED domain and thus abolishing the oligomerisation-stimulated activity of the dynamin may cause haploinsufficiency.

1.7 Is type 1-ADOA really tissue specific?

Even though it is not the only reported case, the tissue specificity of type 1-ADOA is surprising for a disease for which the genetic origin is a nuclear gene encoding a ubiquitously

expressed mitochondrial protein. While most abundant in the retina, the OPA1 mRNA is widely distributed in mammalian tissues (Alexander et al., 2000; Delettre et al., 2000; Pesch et al., 2001). OPA1 protein is not exclusively localized to the RGC in retina since OPA1 immunolabelling was also detected in the outer and inner plexiform layers and in the inner nuclear layer in adult retinal tissues from mammals (Aijaz et al., 2004; Pesch et al., 2004; Ju et al., 2005; Kamei et al., 2005). It should be noted, however, that the presence and subcellular distribution of OPA1 in the optic nerve is somewhat controversial, since OPA1 was not detected in the rat optic nerve (Pesch et al., 2004), though it is highly expressed in the mouse optic nerve axons (Ju et al., 2005). Surprisingly, OPA1 is expressed in the myelinated regions of the human optic nerve, which show a decreased number of mitochondria as compared to the somata of the RGC, where biogenesis of mitochondria occurs, and in the fibre layer containing unmyelinated axons before the lamina cribosa (Aijaz et al., 2004). Since OPA1 is widely expressed in many tissues the reason that RGC are primarily affected by OPA1 mutations remains unknown. On the other hand, recent reports of sensorineural hearing loss associated with dominant optic atrophy caused by the R445H mutation in OPA1 in several unrelated families (Payne et al., 2004; Li et al., 2005; Amati-Bonneau et al., 2003 and 2005) indicate that the RGC are not the only cell types affected in type 1-ADOA.

1.8 Summary and objectives

Mutations in the OPA1 gene cause a degeneration of retinal ganglion cells leading to atrophy of the optic nerve in the patients with autosomal dominant optic atrophy. OPA1 is a ubiquitously expressed mitochondrial protein encoded by a nuclear gene, involved in mitochondrial fusion and cristae remodelling. Delettre and co-workers (2001) reported a splicing hotspot involving exons 4, 4b and 5b that lead to eight different splice variants in human cell. Presence or absence of these exons does not alter the reading frame. In addition, by analogy with the yeast homolog Mgm1p, OPA1 may be post-translationally processed to yield additional isoforms (Herlan et al., 2003), some of which demonstrate different localizations (Satoh et al., 2003). Satoh and co-workers (2003) also reported that OPA1 isoforms build up different complexes in the mitochondrial intermembrane space. Thus it is crucial to clarify the identity and the mechanism by which these isoforms are generated. Moreover, it is important to unravel the function of each of these isoforms and their individual role in maintenance of mitochondrial morphology.

The main focus of our lab is the functional analysis of disease genes affecting the optic nerve, importantly OPA1. One of the major approaches in this respect is the biochemical

characterization of the disease gene products. Therefore, the main focus of my thesis was to characterize of the mouse OPA1 protein at the molecular level, concerning RNA transcript and protein expression.

OBJECTIVES

1. To study the expression of OPA1 splice variants in mouse;
2. To study the expression of OPA1 protein isoforms in mouse;
3. To identify OPA1 interacting proteins.