4. DISCUSSION

4.1. Human and mouse OPA1 transcripts differ mainly in their 3' untranslated region

Transcription of the human OPA1 gene results in the generation of several mRNA species that differ in length by 1 to 3 kb (Alexander et al., 2000). The mouse OPA1 gene is transcribed resulting in two transcript groups differing in length by 2 kb (Figure 9). All *OPA1* transcripts from both human and mouse were here shown to contain all important OPA1-protein domains. Of note, the mitochondrial leader sequence is present in all human and mouse OPA1 transcripts, which excludes the existence of non-mitochondrial, i.e. cytoplasmic, isoforms of OPA1. Human and mouse transcripts do not show crucial differences in their open reading frame, making the mouse genetically a good model to study OPA1 function.

The entire 3'untranslated region of the hOPA1 gene has a length of ~3 kb and exists as one exon according to the genomic data deposited in public databases. A likely mechanism to generate the observed transcript patterns is the differential usage of poly(A) sites in the 3'UTR of the message. Although detailed knowledge of the steps leading to transcriptional termination and polyadenylation are just emerging (Beaudoing et al., 2000; Beaudoing and Gautheret, 2001; Zhang et al., 2005a), three conserved sequence motifs have been defined (Graber et al., 1999). These include: 1. a polyadenylation hexanucleotide signal sequence, AAUAAA in 90% of cases, 10 – 30 bases upstream of the transcript cleavage site, 2. a GU-rich element located 20 to 40 bases downstream of the cleavage site, and 3. a CA dinucleotide immediately 5' to the site of cleavage, which is preferred but not required (Chen and Shyu, 1995). The A-rich sequence element is necessary for the binding of the cleavage/polyadenylation specificity factor (CPSF), whereas the U-rich signal functions as a binding site for the cleavage stimulation factor (CstF) (Zhao et al., 1999). Both interact as part of the core polyadenylation machinery, but more factors are expected to be identified and to take part in the regulation of cleavage site selection and cleavage efficiency (Colgan and Manley, 1997). More than half of the human genes have multiple polyadenylation sites, leading to variable mRNA and protein products (Zhang et al., 2005b). Computer based analysis of EST data led to the detection of the extraordinary high number of 10 putative

poly(A) sites the human OPA1 3'UTR, of which 9 seem to be actively used (Table 2). This is the highest number of alternative poly(A) sites ever described for a gene. The activin βA subunit gene, with 8 possible poly(A) sites and the dihydrofolate reductase (DHFR) gene, with 7 poly(A) sites, currently rank at the top of the list of genes that are known to be differentially polyadenylated (Frayne et al., 1984; Tanimoto et al., 1991). Moreover, the two most frequently used human OPA1 poly(A) sites were found to contain the rather unconventional AUUAAA and AUAAAA hexanucleotides. In contrast, the mouse 3'UTR displayed only 4 possible poly(A) sites, of which just 3 seemed to be actively used including the conserved AUUAAA motif. Finally, this *in silico* predicted polyadenylation clusters were correlated with the individual bands detected on the northern blots, and further emphasised the differential polyadenylation of both human and mouse OPA1 transcripts.

The reason for the need to have more than one OPA1 transcript species available in the cell is not clear. Yet, tissue specific differences in the steady-state levels of the different transcript clusters do exist, which points towards a regulation of poly(A) site selection in connection to OPA1 gene expression. In skeletal muscle, brain and heart, a higher abundance of the long transcript version versus the shorter one was observed, and this was more marked in mouse than in humans. These tissues are typically altered in mitochondrial diseases when mitochondrial dysfunction occurs (Finsterer, 2004; Vogel, 2001).

It is not understood to date, how one poly(A) signal in a given mRNA is chosen from among several, since most studies on transcriptional termination are concentrating on the processing of single poly(A) sites in mRNAs in conjunction with the isolation of co-factors of the basic cleavage/polyadenylation machinery (Orozco et al., 2002; Yeung et al., 1998). Recent findings provide evidence for a connection between transcription, its termination and polyadenylation due to an interaction of components of RNA polymerase II and the mRNA 3'end processing complexes (Proudfoot and O'Sullivan, 2002). In mammalians, the B-cell maturation-dependent class switch of immunoglobulin heavy chains is the only case in which the 64 kDa subunit of CstF was shown to direct poly(A) site choice (Kim et al., 1999; Takagaki and Manley, 1998). However, different modes of control are theoretically possible, like interference of transcriptional termination factors (Vo et al., 2001), which would support the generation of shorter transcripts, as well as anti-termination factors (Wada et al., 1998) promoting read-through to distally located poly(A) sites.

Polyadenylation, per se, influences and promotes translational efficiency after mRNA export from the nucleus (Wickens et al., 1997). In the cytoplasm, transcript stability and individual susceptibility to degradation also play a role and are poly(A)-dependant. The preferential use of a proximal poly(A) site over a more distal one, like shown above for OPA1 mRNAs in mouse liver, will shorten the 3'UTR and remove potentially important regulatory elements necessary for the survival of long OPA1 transcripts in the cytoplasm.

As a consequence, tissue specific polyadenylation of OPA1 transcripts may regulate levels of OPA1 protein. This question could now be finally answered by expressing different transcripts separately in mouse embryonic fibroblasts (MEFs) lacking OPA1.

4.2. Reduced levels of OPA1 protein do not lead to retinal ganglion cell death in mice

In the OPA1$^{+/-}$ mice, disruption of one *OPA1* gene copy leads to reduced levels of OPA1 protein in various tissues (Figure 13). This means that limited *OPA1* gene dosage in OPA1$^{+/-}$ mice can not be compensated for by increased gene expression of the healthy allele in order to restore OPA1 protein wild-type levels. An extensive analysis of the visual system was performed, but no defect of either the retinal ganglion cells (RGCs) or the optic nerve in OPA1$^{+/-}$ mice was observed in up to 1-year-old animals. Besides the histological studies of retina and optic nerve, other structural and functional studies of the visual and hearing system were performed. In collaboration with Mathias W. Seeliger, Tübingen, a potential loss of the RGCs in the eyes of the OPA1$^{+/-}$ mice was examined using scanning laser ophthalmoscopy (SLO). This method facilitates confocal imaging of the retinal layers *in vivo* (Seeliger et al., 2005). Lasers with different wavelengths obtain information about specific retinal layers due to their reflection and transmission characteristics and, thus, the numbers and densities of RGC fibers can be examined. OPA1 heterozygous knockout mice of different stages (6 weeks, 3 and 12 months old) did not show any changes in fiber number and density compared to wild-type mice (Figure 25 A). There were no signs of increased auto-fluorescence as commonly observed in apoptotic cell death (Seeliger et al., 2005). In the same laboratory, electroretinogram (ERG) recordings were performed, in order to test functional properties of the retina. This

is a physiological, non-invasive technique that is commonly used for diagnostic purposes in human patients (Marmor et al., 2004) and mouse models (Seeliger et al., 2001). The recorded signals reflect mainly the sum of rod- and cone-system responses to light (though bipolar cell signals are also seen) and allow to objectively assess visual function in the retina. The ERG of heterozygous mice did not differ in any way from the recordings obtained from their wild-type littermates, indicating functionally intact retinae (Figure 25C-F).

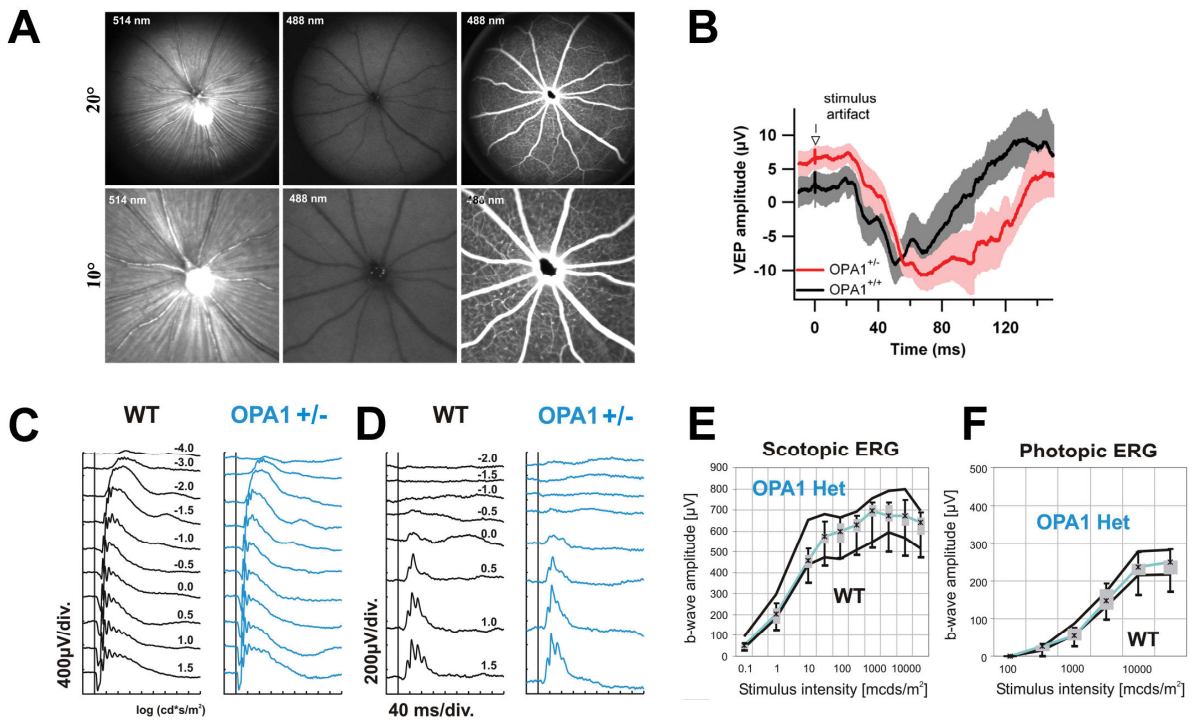


Figure 25. Analysis of visual system of OPA1+/- versus OPA1+/+ mice

(A) Scanning laser ophthalmoscopy (SLO) of retinae of OPA1$^{+/-}$ mice *in vivo*. SLO confocal imaging allows visualising the nerve fiber layer and its potential reduction due to loss of retinal ganglion cells (RGC). OPA1$^{+/-}$ mice did not show any changes in fiber number and density compared to wild-type mice (for wild-type see: Seeliger et al., 2005). (B) Visual evoked potentials (VEP) from OPA1$^{+/-}$ heterozygous mice and wild-type littermates (n=8 each). (C-F) Scotopic (dark adapted) and photopic (light adapted) electroretinogram (ERG) recordings performed on wild-type and heterozygous mice. The recorded signals reflect the sum of rod and cone system responses to light and allowed to objectively assess visual function on a retinal level. (C) Scotopic single flash intensity series. (D) Photopic single flash intensity series. (E) Scotopic b-wave amplitude vs. log intensity function. Boxes indicate the 25% and 75% quantile range, whiskers indicate the 5% and 95% quantile and the asterisk indicates the median of the OPA1$^{+/-}$ data. The normal range was delimited by solid lines indicating the 5% and 95% quantile of the wild-type data. (F) Photopic b-wave amplitude vs. log intensity function. No sign of impaired retinal function was found in the heterozygous mice.

Recordings of visually evoked potentials (VEP), which reflect synchronous activation of the optic cortex, were performed by N. Strenzke, in collaboration with T. Moser, Göttingen, in order to assess functionality of the optic nerve (Eiberger et al., 2006). VEPs recorded from 6-week-old OPA1$^{+/-}$ mice and control littermates did not reveal impaired neural transmission of information from eye to visual cortex in the mutant mice (Figure 25B). Besides, auditory brainstem responses (ABR) were measured in the same laboratory. ABR reflect the synchronized electrical activity of the auditory pathway, and thus provide information on the sequential processing of acoustical signals in the inner ear and brainstem (Eiberger et al., 2006). Hence, they depend on intact function of the cochlea, auditory nerve and auditory brainstem. ABR from OPA1 heterozygous mice showed normal amplitudes, latencies and hearing thresholds over a wide range of stimulus frequencies and intensities (Figure 26). Distortion product otoacoustic emissions (DPOAE) were also measured. They reflect the function of the active preneural elements, namely cochlear amplifier, that make the initial contribution to the perception of acoustic stimuli (Lonsbury-Martin and Martin, 1990). DPOAEs represent one type of evoked emission that has significance as an important test in the audiometric evaluation of hearing capacity. In OPA1$^{+/-}$ mice level and amplitude functions for DPOAE were also found to be normal (not shown).

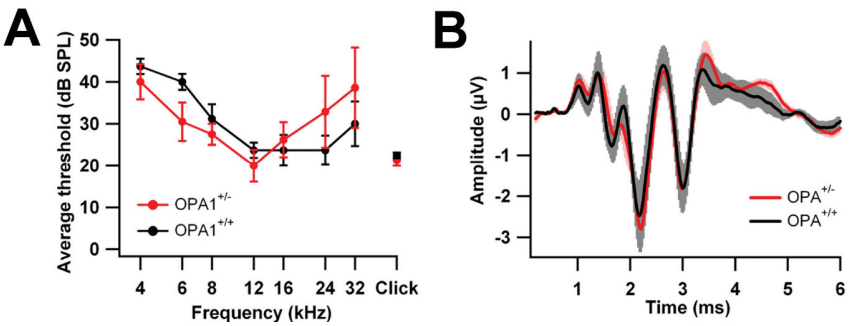


Figure 26. Analysis of acoustic system of OPA1+/- versus OPA1+/+ mice

(A) Hearing thresholds ± SEM as determined by auditory brainstem responses (ABR) recordings in response to tone bursts or click stimuli and (B) mean ABR ± SEM to 60 dB click stimuli from OPA1$^{+/-}$ heterozygous mice and wild-type littermates. n=8 each.

4.3. Speculations on how OPA1 mutations may lead to adOA

adOA patients suffer from selective impairment of vision, while the entire rest of their nervous system functions normally (Kjer, 1959). As discussed above, OPA1 is widely expressed throughout the body (Delettre et al., 2001), but only retinal ganglion cells are found to be affected (Kjer, 1959). More recently, sensorineuronal hearing loss associated with dominant optic atrophy was connected with the particular point mutation R445H (Amati-Bonneau et al., 2003; Li et al., 2005; Payne et al., 2004). It has been shown in many publications and in this thesis, that OPA1 plays important roles for the normal function of mitochondria. Thus, mitochondrial dysfunctions caused by mutations in OPA1 lead to adOA. Besides, fibroblasts from patients bearing the R445H allele were shown to contain highly fragmented mitochondria (Amati-Bonneau et al., 2005). Retinal ganglion cells (RGC) are far from being the only cells requiring functional mitochondria. So, what renders them so uniquely vulnerable to mitochondrial dysfunction? Axons of RGCs show long unmyelinated stretches that have much slower conduction velocities and require far greater energy to restore the electrical potential. This makes them bioenergetically demanding elements of the central nervous system (Carelli et al., 2004a). These unmyelinated regions are, therefore, enriched with mitochondria. Furthermore, the relative oxygen consumption in the retina is even higher than in the brain, being one of the highest oxygen consuming tissues in the body (Yu and Cringle, 2001). Finally, biogenesis of mitochondria takes place within the RGC soma, from where they have to be transported through the optic nerve in order to be distributed along energy-dependent locations, like many unmyelinated regions, nodes of Ranvier and the synaptic terminals (Grafstein, 1969). The local need for ATP and Ca^{2+} is particularly high at synapses, and mitochondria are therefore frequently found in axon terminals (Palay, 1956; Rowland et al., 2000; Shepherd and Harris, 1998). It has been proposed that dendritic distribution of mitochondria, which is regulated by Drp1, OPA1 and neuronal activity, could be an essential and a limiting factor for synapse density and plasticity (Li et al., 2004). Therefore, abnormalities in mitochondria may lead to local functional and metabolic crises as a result of local energy depletion.

So, the first explanation for an adOA retinal-specific phenotype could be that the loss of one allele decreases the amount of OPA1 protein below a critical threshold for normal functionality of mitochondria in tissues that have an extra high demand for energy.

This could also be the explanation for another retinal mitochondriopathy, LHON (Carelli et al., 2004a), that shares some clinical symptoms with adOA (Jacobson and Stone, 1991), but is caused by mutations in the mtDNA (Erickson, 1972).

A second possibility could be that certain environmental factors influence the RGC susceptibility to disease. Daily exposure of the retina to UV light might be the trigger for increased apoptosis in cells that carry mutations in OPA1. In adOA, RGC degeneration is most likely due to apoptosis, since OPA1 has a very important role in apoptosis. Preliminary results reported in Olichon et al. (Olichon et al., 2006) showed that skin fibroblasts of some adOA patients exhibited an increased sensitivity to staurosporine-induced cell death. In experiments with LHON cybrid cells, it was shown that these cells when put under stress are sensitised to an apoptotic death through a mechanism involving mitochondria (Ghelli et al., 2003; Zanna et al., 2005).

4.4. Why do OPA1 heterozygous knockout mice not show the same phenotype as human carriers of OPA1 mutations?

Disruption of one *OPA1* gene copy leads to reduced levels of OPA1 protein in various tissues of the $OPA1^{+/-}$ mice. In humans, adOA is thought to be caused by haploinsufficiency of OPA1. Thus, it is most likely the case that OPA1-protein levels are diminished throughout the bodies of adOA patients, while only the RGCs (and in rare cases the ear) become vulnerable to this situation. Decreased levels of OPA1-protein correlate with loss of mtDNA in $OPA1^{+/-}$ mice and in adOA patients (Kim et al., 2005). Furthermore, mouse and human *OPA1* show similar expression. In humans, first symptoms of adOA appear already in the first decade of life. So, why are $OPA1^{+/-}$ mice spared from similar symptoms?

Progressive neurodegeneration with manifestation in adult life, as in Alzheimer's and Parkinson's disease has proven difficult to model in mice (Gotz et al., 2006; Shimohama et al., 2003). The reason may be that overt clinical defects will not occur in the short life span of mice (3 years), and molecular makers of the initial neurodegenerative process are unknown (Wynshaw-Boris, 1996).

Another explanation for the lack of disease-like symptoms in $OPA1^{+/-}$ mice could be that the eyes of humans and mice have fundamental anatomical differences. In particular,

mice do not possess an equivalent to the human fovea, the central point of vision characterised by extremely dense packing of photoreceptors and retinal ganglion cells (Smith, 2002). The sense of vision altogether is not as important for mice as it is for man. As a consequence, the visual cortex in mice is rather rudimentarily developed (Hubener, 2003).

Further, it may well be that the genetic alteration alone is not sufficient to produce pathological changes, but additional factors could be necessary to trigger optic nerve degeneration in OPA1$^{+/-}$ mice. For example, nutritional restrictions or UV light exposure could provide environmental conditions influencing the survival potential of retinal ganglion cells (de Oliveira Miguel et al., 2003). Evidence for such a phenomenon was reported from Cuba in 1992, where many people were suffering from an epidemic of optic neuropathy (Sadun et al., 1994). Application of vitamin A and B had a great effect in improving the patients' conditions. Besides, it is known for many other mitochondriopathies that environmental conditions can increase susceptibility of neurons to the disease. E.g. in LHON, tobacco smoking and alcohol consumption (Carelli et al., 2002a; Newman, 1978) as well as many other toxins (Carelli et al., 2004b; Sadun and Carelli, 2003) have been described to induce disease symptoms.

Lastly, analysis of affected adOA families demonstrated that the disease phenotype is not fully penetrant, indicating that modifier loci exist (Hoyt, 1980; Johnston et al., 1999; Votruba et al., 1998). It remains to be established if changes in vision and hearing can be observed in mice if the OPA1 mutation is present on other genetic backgrounds.

4.5. Loss of OPA1 leads to early mammalian embryonic death

The OPA1 knockout mouse model described in this thesis represents the first tool that allows assessment of OPA1 function *in vivo* in a mammalian organism. OPA1 was proven to be absolutely essential for early embryonic development, because embryos lacking both gene copies die during early gastrulation at E7.5 to E8.5. The exact stage when death occurs is likely to depend on the genetic background, as no surviving embryos were observed on a mixed C57BL/6/129/Ola or pure 129/Ola background after E8.5, whereas embryos survived to E9.5 on a mixed Black Swiss/C57BL/6/129/Ola

background. At E8.5, the embryo has already implanted into the uterus, but a placenta has not yet formed. Therefore, one can exclude that OPA1$^{-/-}$ mice die due to placental defects, as observed for Mfn2$^{-/-}$ mice (Chen et al., 2003).

Homozygous carriers of human OPA1 mutations have never been described. Since complete knockout of OPA1 in the mouse is lethal, this mouse model provides evidence for the fact that complete loss of OPA1 is incompatible with life and inhibits mammalian embryonic development. It seems as if one functional OPA1 gene copy is absolutely necessary for survival in both mice and man.

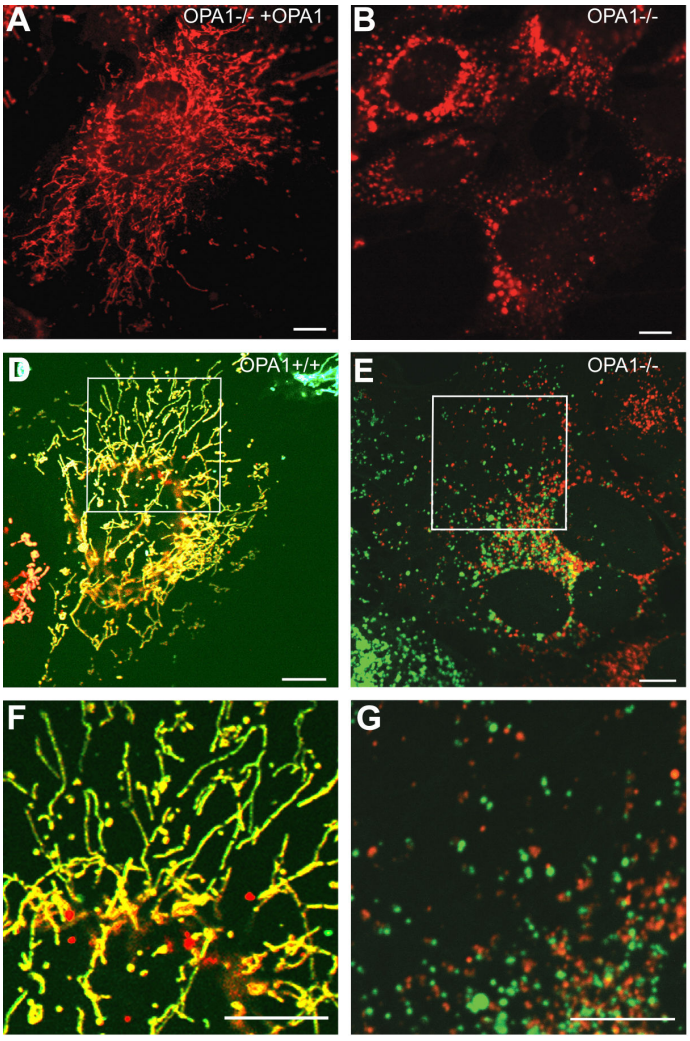
4.6. Decrease of mtDNA levels in OPA1$^{-/-}$ embryos

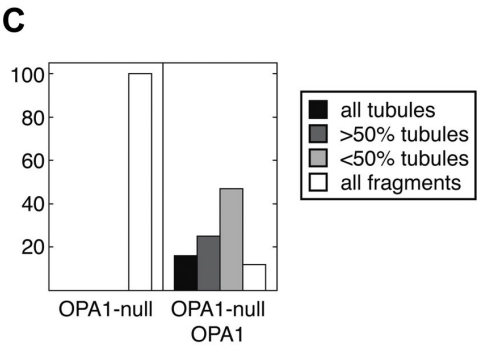
Mitochondrial DNA codes for the most hydrophobic subunits of the different respiratory chain complexes. mtDNA mutations or mutations in genes affecting mtDNA stability or copy number (Howell et al., 2005; Leonard et al., 2002) lead to a loss of mtDNA, which results in reductions in oxidative phosphorylation (Smeitink et al., 2001). Shortage in energy supply is often the cause for mitochondrial metabolic disorders. Tfam (mitochondrial transcription factor A) and RNaseH1 are proteins known to regulate mtDNA copy number (Cerritelli et al., 2003; Larsson et al., 1998). Minor reductions of mtDNA levels of up to 35% in both heterozygous Tfam and RNaseH1 deficient animals did not have any effect on survival (Ekstrand et al., 2004). Even complete loss of mtDNA in both Tfam and RNaseH1 knockout animals, did not lead to apoptosis and death of embryos before E8.5 (Cerritelli et al., 2003; Larsson et al., 1998). OPA1-null cells do suffer from great impairment of respiratory chain activity. On the other hand, OPA1$^{-/-}$ embryos die at E8.5, just like Tfam$^{-/-}$ and RNaseH1$^{-/-}$ embryos, albeit containing 60% of wild-type mtDNA levels (Figure 17). Therefore, one can conclude that they contain enough mtDNA molecules for survival.

4.7. Cells devoid of OPA1 have lost the ability to fuse their mitochondria

Balancing between mitochondrial fusion and fission is a way for eukaryotic cells to maintain the overall shape of their mitochondria. Unbalanced fission leads to

mitochondrial fragmentation and unbalanced fusion to mitochondrial elongation (Legros et al., 2002). These processes are not only implicated in mitochondrial shaping but also in the control of mitochondrial function. Without these dynamics the mitochondrial population consists of autonomous organelles that might have impaired function. Mitochondrial dynamics actually allows mitochondria to interact with each other. Fusion mediates molecular exchanges between mitochondria (Legros et al., 2002). The connections between mitochondria facilitate energy transmission between different cellular regions (Amchenkova et al., 1988), transfer of the mitochondrial membrane potential from oxygen-rich to oxygen-poor cellular regions (Skulachev, 2001) and determines the size and dynamics of the mitochondrial Ca^{2+} pools (Collins et al., 2002; De Giorgi et al., 2000; Park et al., 2001; Rizzuto et al., 1998). In addition, the efficient fusion-mediated exchange between individual mitochondria allows complementation of different mtDNA pools, i.e. mutated with wild-type mtDNA molecules (Ono et al., 2001). It has been shown that disruption of fusion caused by knocking out of mitofusins (Mfn1 and Mfn2) leads to severe defects in mice (Chen et al., 2003). Mfn1 and Mfn2 proteins are, like OPA1, large mitochondrial GTPases (Ishihara et al., 2003; Santel and Fuller, 2001), and their complete knockout is embryonic lethal, probably due to their crucial role in mitochondrial fusion (Chen et al., 2003). Mouse embryonic fibroblasts (MEFs) derived from $Mfn1^{-/-}$ and $Mfn2^{-/-}$ embryos contained fragmented mitochondrial networks (fragmentation was found to be greater in $Mfn1^{-/-}$ cells) and showed impaired fusion (Chen et al., 2005; Chen et al., 2003) but the corresponding embryos survived at least until E11.5 and E10.5, respectively (Chen et al., 2003). In contrast, $OPA1^{-/-}$ embryos contained only fragmented mitochondria, and even $OPA1^{+/-}$ embryos had a certain fraction of fragmented mitochondria, whereas wild-type embryos displayed only tubular mitochondria. The degree of OPA1 loss in the mouse embryo, either a slight reduction of protein in $OPA1^{+/-}$ mice or a complete loss in $OPA1^{-/-}$ mice was mirrored by the extent of mitochondrial fragmentation and, even more interestingly, was correlated with cell survival. Further, mitochondria of both $OPA1^{-/-}$ (Figure 27) and double $Mfn1/Mfn2^{-/-}$ cells (Chen et al., 2005) were totally unable to fuse, unlike $Mfn1^{-/-}$ and $Mfn2^{-/-}$ cells that did not show a complete loss of fusion. In addition, Mfn1/Mfn2 double mutant embryos and $OPA1^{-/-}$ embryos, if on mixed Black Swiss/C57BL/6/129/Ola background, died around E9.5 whereas $Mfn1^{-/-}$ and $Mfn2^{-/-}$ embryos survived longer.





H

Cells R x G	Fusion: Full	Partial	Very little	No
+/+ x +/+	188	12	0	0
-/- x +/+	2	194	3	1
-/- x -/-	0	0	0	200
+/- x +/-	183	16	1	0

Figure 27. Mitochondrial fragmentation and lack of fusion in OPA1-null fibroblasts

(A and B) Confocal image of MitoTracker Red-stained fibroblasts. (A) After expression of OPA1 isoform 1 by retroviral transduction, most cells contain a substantial population of tubular mitochondria and look like wild-type. This rescue of mitochondrial tubules is quantitated in (C). (B) In OPA1-null fibroblasts all mitochondria are fragmented. (D, F) Mitochondrial fusion in a wild-type polyclonal cell. (E, G) Lack of mitochondrial fusion in a cell hybrid of OPA1 null cells. Scale bar: 10 µm. F and G are a close-up of the boxed regions in D and E, respectively. (H) A quantification of the fusion events in the PEG fusion assay of red (R) and green (G) labelled mitochondria in fibroblasts of indicated genotypes (+/+ stands for wild-type, -/- for OPA1$^{-/-}$ and +/- for OPA1$^{+/-}$ mouse embryonic fibroblasts).

These results lead to one simple conclusion: Cells lacking mitochondrial fusion proteins, like Mfn1, Mfn2 and OPA1 are unable to fuse their mitochondria and are committed to die, most likely due to inability of their mitochondria to cooperate with each other. In such cells, any potential defects in mitochondrial function cannot be complemented by fusion of affected mitochondria with healthy mitochondria. Loss of OPA1 produced a greater fusion incompetence than elimination of either of the mitofusins and, thereby, led to an earlier embryonic death, which was similar to the phenotype observed in mitofusin-double knockout mice. Whether cell death in the absence of OPA1 is directly or indirectly connected to the role of OPA1 in fusion is still a matter of debate and remains to be elucidated.

4.8. Connection between fragmented mitochondrial phenotype of OPA1 knockout embryos and apoptosis

Mitochondria are central players in the initiation and execution of apoptosis (Kroemer and Reed, 2000). Mitochondrial dysfunction in cell death is characterised by a decline in mitochondrial membrane potential ($\Delta\Psi_m$), respiratory defects, an increase in reactive oxygen species (ROS) production, changes in ATP levels and release of apoptogenic factors including cytochrome c and apoptosis inducing factor (AIF). Early during apoptosis the mitochondrial network undergoes fragmentation and this is coinciding with cytochrome c release (De Vos et al., 1998; Desagher et al., 1999; Frank et al., 2001; Karbowski et al., 2002; Karbowski and Youle, 2003; Zhuang et al., 1998). A set of evidences suggests that fission is an early step during apoptosis. Dynamin-related protein (Drp1) and hFis1 are proteins involved in mitochondrial fission (Hinshaw, 2000; James et al., 2003; McNiven et al., 2000; Smirnova et al., 2001; Smirnova et al., 1998; van der Bliek, 1999; Yoon et al., 2003). Drp1 is a large cytosolic GTPase that translocates to the mitochondria during fission, coupling GTP hydrolysis with scission of the mitochondrial tubule (Pitts et al., 1999; Smirnova et al., 2001; Smirnova et al., 1998). hFis1 is thought to be its receptor at the surface of the mitochondria, anchored to the outer mitochondrial membrane facing the cytoplasm (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003; Yu et al., 2005). Blocking mitochondrial fission by overexpression of a dominant negative form of Drp1, $Drp1_{K38H}$, prevents apoptosis (Frank

et al., 2001). Overexpression of hFis1 results in cytochrome c release and cell death (James et al., 2003; Yoon et al., 2003) and its downregulation by RNA interference prevents apoptosis (Lee et al., 2004). At the same time, Mfn1-dependent mitochondrial fusion is largely inhibited in the early course of cell death (Karbowski et al., 2004), and combined overexpression of rat Fzo1A,B (homologues of human Mfn1 and 2) protected from cell death by intrinsic apoptosis-inducing stimuli (Sugioka et al., 2004).

Fragmentation does not necessarily lead to programmed cell death (Lee et al., 2004). Drp1 was able to inhibit apoptosis induced by oxidative stress and ceramide (Szabadkai et al., 2004). Ceramide causes the release of Ca^{2+} from endoplasmatic reticulum (ER) stores (Pinton et al., 2001) and sensitises mitochondria to Ca^{2+}-induced permeability transition pore complex (PTPC) opening (Pacher and Hajnoczky, 2001). This pore is known to open upon accumulation of Ca^{2+} in the mitochondrial matrix (Bernardi, 1992). Similarly, ROS can lower the threshold of Ca^{2+} required for PTPC opening (Brookes, 2005). High mitochondrial Ca^{2+} concentration can, in the presence of such stimuli, induce mitochondrial outer membrane permeabilisation (MOMP) in a process that proceeds as a wave, while Drp1-induced fragmentation, on the other hand, can interrupt intramitochondrial Ca^{2+} waves and can reduce mitochondrial Ca^{2+} uptake. Secondly, mammalian cells treated with protonophores, which dissipate $\Delta\Psi_m$, and thus, inhibit fusion, display heavily fragmented mitochondria but remain viable for up to 24 h, while their mitochondria retain cytochrome c (Legros et al., 2002; Lim et al., 2001; Smaili et al., 2001).

$OPA1^{-/-}$ embryos contain mitochondria that seem to be locked in the fragmented state, are unable to fuse and have disorganised cristae. It might be that, as commented above, those cells are unable to complement mitochondrial defects by fusion with healthy mitochondria, which eventually leads to the induction of apoptosis. This is in agreement with results from *in vitro* experiments where OPA1 siRNA triggered fragmentation of the mitochondrial network and apoptosis (Olichon et al., 2003). Mitochondria of these cells, as well as mitochondria of $OPA1^{-/-}$ MEFs, present disrupted cristae structures. Germain and colleagues (Germain et al., 2005) showed Drp1-dependent remodelling of the inner membrane during apoptosis, establishing for the first time a relationship between changes in the morphology of the mitochondrial reticulum and remodelling of cristae. Dominant negative Drp_{K38E} was able to inhibit the remodelling of the cristae following apoptosis induction. If fission is associated with cristae remodelling, fragmented mitochondria may

be prompted for maximal release of cytochrome c. On the other hand, Frezza and colleagues (Frezza et al., 2006) showed that OPA1 controls apoptotic cristae remodelling and state that this was independent from the role of OPA1 in fusion. Overexpression of isoform 1 of OPA1 was found to prevent apoptosis in $Mfn1^{-/-}$ and $Mfn2^{-/-}$ cells but could not rescue the fragmented morphology of their mitochondria. Besides, changes in cristae organisation of *Mgm1*-deleted yeast (yeast *OPA1* homologue) were reversible after *Dnm1* (yeast *Drp1* homologue) inactivation. Since Dnm1/Drp1 is a fission promoting and Mgm1/OPA1 a fusion promoting protein, the role of Mgm1/OPA1 in the structural organisation of the cristae may be an indirect effect of its function in mitochondrial fusion (Sesaki et al., 2003). The story becomes even more complicated knowing that overexpression of the same OPA1 isoform in some cases leads to elongation of mitochondria in HeLa cells and MEFs (Cipolat et al., 2004; Olichon et al., 2002), whereas in other cases mitochondria fragment in HeLa and COS-7 cells (Griparic et al., 2004; Misaka et al., 2002).

Besides changes in mitochondrial morphology, MOMP is a critical event in the process of programmed cell death, which is induced by a subclass of proapoptotic Bcl-2 proteins like Bax and Bak (Cory and Adams, 2002; Wei et al., 2001). These proteins seem to be in an inactive state in healthy cells, with Bax being mostly found in the cytosol. However, during apoptosis they are activated by a process requiring BH3-only Bcl-2 family members (Bouchier-Hayes et al., 2005; Huang and Strasser, 2000). One of the consequences of this process is oligomerisation and insertion of Bax and Bak into the outer mitochondrial membrane, which results in MOMP (Antonsson et al., 1997; Schlesinger et al., 1997). It has been shown that Bax colocalises with Drp1 and Mfn2 near to “scission foci” of ongoing mitochondrial fission events during apoptosis (Karbowski et al., 2002; Neuspiel et al., 2005). However, there is no evidence in the literature showing a direct interaction between Bax and Drp1 or Mfn2, though such interaction might exist.

Pro-apoptotic signals influence the connectivity of the mitochondrial network and tend to induce its fragmentation before, during or after induction of MOMP (Perfettini et al., 2005). Whether fission and/or disruption of fusion leads to apoptosis, or fission is just a by-product of apoptosis induction is unclear. There is evidence for the involvement of both pathways, showing the necessity of further investigation in this field. Surely, OPA1 plays a very important role in both fusion of mitochondria and apoptosis and both processes are affected in OPA1 knockout embryos.

4.9. OPA1 is required for cristae maintenance, oxidative phosphorylation and mitochondrial membrane potential

Mitochondria are organelles surrounded by double-membrane. The inner membrane is complex and folded into invaginations called cristae. They represent places where cytochrome c and the majority of the respiratory chain complexes localise to (D'Herde et al., 2000; Perotti et al., 1983). Cristae are separate compartments connected to the thin intermembrane space by narrow tubular junctions (Frey and Mannella, 2000), the cristae junctions. Electron microscopic (EM) analysis of $OPA1^{-/-}$ MEF cells revealed completely disorganised mitochondrial cristae (Figure 20), demonstrating a role for OPA1 in maintaining organised inner mitochondrial membranes. In $OPA1^{-/-}$ MEFs, cristae appeared like septae or like vesicles that had lost connection to the inner membrane. In order to evaluate the functionality of mitochondria containing such abnormal membranes, mitochondrial membrane potential and respiration in $OPA1^{-/-}$ MEFs were examined. In accordance with previously published OPA1 RNAi data (Chen et al., 2005; Olichon et al., 2003), $OPA1^{-/-}$ MEFs were respiration deficient and showed dramatically reduced membrane potential ($\Delta\Psi_m$). Complex IV activity was impaired, while the protein level of complex I was slightly reduced. A direct role of OPA1 in control of respiratory chain complexes has never been implicated and a more likely explanation for this severe reduction of mitochondrial respiration may be an indirect effect on large respiratory chain complexes due to disorganised cristae.

In normal cells the mitochondrial membrane potential is mainly maintained by transmembrane proton pumping occurring during electron transfer by respiratory chain complexes (Lodish, 2004). It has been shown that cell lines devoid of mtDNA (ρ0 cells) are respiration deficient but are still able to build up an electrical membrane potential. The $\Delta\Psi_m$ in ρ0 cells is generated via cytoplasmic ATP hydrolysis by F1 ATPase and ATP^{4-} /ADP^{3-} exchange through ANT (adenine nucleotide translocator) located in the inner mitochondrial membrane (Buchet and Godinot, 1998; Kolarov and Klingenberg, 1974). These cells show efficient fusion proving the fact that functional respiratory chain is not a requirement for mitochondrial fusion (Legros et al., 2002). Mitochondria in $OPA1^{-/-}$ cells

seem to be locked in the fragmented state since both respiration as well as ATP synthase-mediated build up of $\Delta\Psi_m$ is affected.

Loss of OPA1 leads to an early embryonic death and this could be due to insufficient mitochondrial respiration. Mammalian development depends on respiration. For instance, mice deficient for mtDNA replication or cytochrome c suffer from impaired respiratory chain activity and early embryonic lethality (Cerritelli et al., 2003; Larsson et al., 1998; Li et al., 2000). Although levels of mtDNA in the OPA1$^{-/-}$ fibroblasts as well as OPA1$^{-/-}$ embryos were diminished, OPA1$^{-/-}$ embryos do still contain enough mtDNA molecules for survival. Interestingly, MEFs that originated from cytochrome c-deficient embryos are resistant to various types of apoptotic stimuli (staurosporine, UV irradiation, serum starvation). Thus, apoptosis is most likely not the cause of early lethality in cytochrome c knockout mice. Cytochrome c$^{-/-}$ MEFs are defective in cell growth and mitochondrial respiration and somewhat resemble OPA1$^{-/-}$ MEFs. Therefore, the death observed in OPA1$^{-/-}$ and cytochrome c$^{-/-}$ embryos could rather be due to impaired respiratory complex activity.

4.10. OPA1 is needed for the activation of apoptosis via mitochondria

Stimulation of apoptosis in OPA1 wild-type, heterozygous and homozygous MEFs by staurosporine was performed in order to further test the role of OPA1 in apoptosis. Interestingly, OPA1$^{-/-}$ MEFs were less sensitive to this stimulation than wild-type or heterozygous MEFs. This tells us that OPA1 is required for the induction of apoptosis via mitochondria. All previously published work provided evidence for rather antiapoptotic role of OPA1 after induction of apoptosis (staurosporine, H_2O_2, etopside, UV light). Two groups induced rapid cytochrome c release by OPA1 siRNA (Arnoult et al., 2005; Olichon et al., 2003) and Frezza and colleagues (Frezza et al., 2006) were able to prevent apoptosis by overexpression of isoform 1 of OPA1. At a first glance, these results are contradictory to the finding presented here. The complete lack of OPA1 in OPA1$^{-/-}$ MEFs reduced staurosporine-induced apoptosis (Figure 23). The explanation for this observation may lie in the differences in the various experimental systems used to examine OPA1's role in apoptosis. In the siRNA experiments, cells still contain residual amounts of OPA1, which may be enough to drive these cells into apoptosis.

In overexpression experiments, only one out of eight possible OPA1 isoforms was used, presumably the isoform implicated in apoptosis protection. It needs to be stressed that other endogenous isoforms are still present in those cells in physiological amounts. In contrast, cells studied in this work are completely devoid of all endogenous OPA1. Interestingly, EM pictures of OPA1$^{-/-}$ cells revealed mitochondrial cristae (Figure 20) that resemble cristae of cells overexpressing OPA1 (Frezza et al., 2006) and not of OPA1-RNAi cells. Besides, the same group has shown that OPA1 is, indeed, involved in the release of cytochrome c during apoptosis (Cipolat et al., 2006). One cannot exclude, however, that the introduction of large T antigen into the OPA1$^{-/-}$ fibroblasts might have interfered with signalling pathways involved in the induction of apoptosis.

Staurosporine was shown to induce apoptosis of melanoma and leukaemia cells by two pathways (Belmokhtar et al., 2001; Zhang et al., 2004). The first is an early, caspase-dependent pathway induced by cytochrome c release. The second is a late apoptotic event, a caspase-independent pathway and it is induced by the apoptosis-inducing factor (AIF), which is translocated from mitochondria into the nucleus. OPA1$^{-/-}$ cells might be unable to activate caspase-dependent pathways and apoptose later due to the caspase-independent pathways. This idea is also supported by the finding presented in this thesis that OPA1$^{-/-}$ MEFs are unable to release cytochrome c after apoptosis induction, unlike the wild-type MEFs, which do release the cytochrome c and undergo apoptosis (Figure 23). Additionally, lack of OPA1 did not affect cytochrome c-independent apoptotic pathways induced by TNF- α.

If OPA1 is needed for cytochrome c release and activation of caspase-dependent apoptosis, then how can OPA1$^{-/-}$ embryos undergo apoptosis? It might be that in embryos residual maternal OPA1 is sufficient to drive the cells into apoptosis. This could also explain the strong apoptotic phenotype in OPA1-RNAi cells (Arnoult et al., 2005; Olichon et al., 2003). It may be that, maternal OPA1 in the embryos is sufficient to lead mitochondrial fusion-incompetent and respiration-deficient cells into apoptosis. Finally, this massive apoptosis in OPA1$^{-/-}$ embryos may eventually lead to their death.

4.11. OPA1 isoform 1 was able to rescue respiration and fusion but not apoptosis phenotype in OPA1$^{-/-}$ cells

The various roles of OPA1 in cristae remodelling, mitochondrial fusion and cytochrome c release might depend on individual isoforms and/or processed forms of OPA1. OPA1 knockout animals cannot help to dissect these different functions, since all forms of OPA1 are simultaneously deleted in these mice. On the other hand, the OPA1$^{-/-}$ MEFs represent a perfect tool to study these distinct functions of OPA1 isoforms individually. The first example was presented here, where OPA1$^{-/-}$ cells were stably transduced with the OPA1 isoform 1. OPA1 isoform 1 alone could rescue the tubular shape of the mitochondrial network (Figure 27A). But not all mitochondria had retrieved tubular shape, either due to transduction inefficiency, or due to the usage of only one isoform. These restored mitochondrial networks are shown in this thesis to be functional in several aspects. The activity of respiratory complex IV was completely restored and the mitochondrial membrane potential was elevated to wild-type levels. These results speak in favour of OPA1 isoform 1 as a major factor for mitochondrial cristae organisation and mitochondrial fusion.

Not all phenotypic changes in OPA1$^{-/-}$ cells were restored to wild-type characteristics by introducing OPA1 isoform 1. Even with isoform 1 present, cells remained insensitive to staurosporine. This isoform was suggested recently to have an apoptosis-protective role (Frezza et al., 2006). The model proposed by Frezza et al. suggests that OPA1 regulates cytochrome c release from mitochondria by directly opening the cristae junctions. In contrast, data presented in this thesis support a situation in which cristae junctions are blocked from opening due to the loss of OPA1 (Figure 28). Still, this does not exclude a function of OPA1 in indirectly facilitating the opening of cristae. OPA1 contains a GTPase domain, and its ability to open cristae junctions might correlate with the presence in a GTP- or GDP-bound state. Alternatively, other isoforms might work together with the isoform 1 in remodelling of the inner membrane/cristae required for release of cytochrome c and apoptosis.

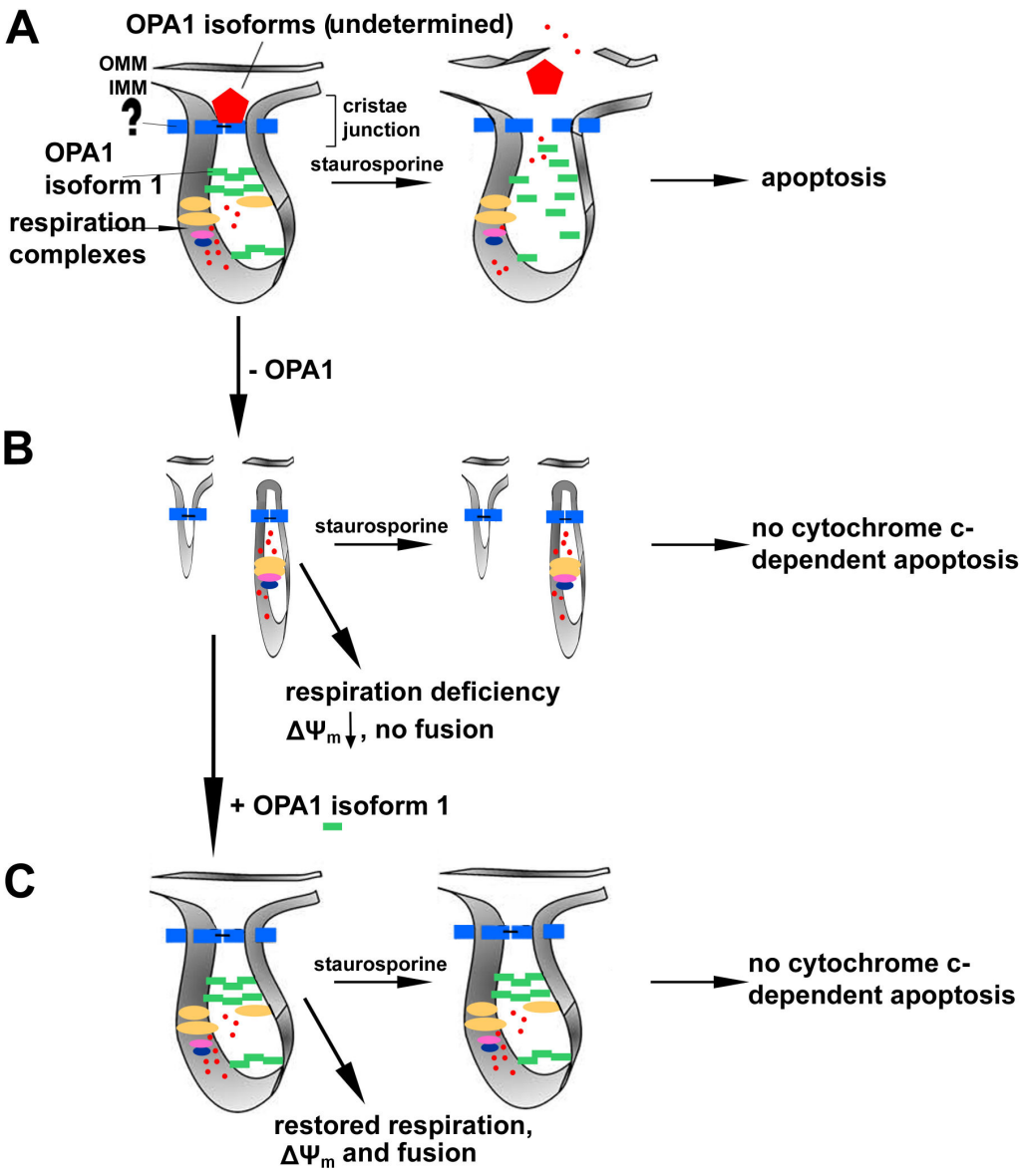


Figure 28. Hypothesis on function of OPA1 deduced form MEF-studies

(A) In wild-type cells induction of apoptosis (staurosporine) leads to opening of cristae junctions depending on OPA1 action. Which OPA1 isoforms are involved in this process is still unclear, but combined action of different isoforms is not excluded. **?** represents an unknown interlock molecule that may keep the cristae junctions tight and works together with OPA1 to open them. (B) Complete knockout of OPA1 disrupts cristae structure and leads to respiration deficiency. Whether OPA1 has a direct or indirect role in these processes remains to be dissolved. Induction of apoptosis in these cells does not lead to cytochrome c release since OPA1, as cristae-opener is not present. (C) Introduction of OPA1 isoform 1 into $OPA1^{-/-}$ cells restores respiration but not the sensitivity to staurosporine. Isoform 1 of OPA1 is necessary and sufficient for proper mitochondrial respiration but not for opening of cristae junctions. This could be due to its anti-apoptotic function or due to absence of other OPA1 isoforms, which may function together in opening of cristae after apoptosis induction. OMM and IMM indicate outer and inner mitochondrial membrane, respectively.

4.12. Importance and future prospects

OPA1 plays a crucial role in cells due to its importance for various mitochondrial functions, especially during early embryonic development. This work shows for the first time that OPA1 is necessary for mammalian cell survival during development. One can postulate a possible scenario of the early embryonic death in $OPA1^{-/-}$ mice. It could be that, together with other maternal components of the oocyte, maternal OPA1 protein begins to fade out by E6.5, so that $OPA1^{-/-}$ mice last just until E8.5. Since the loss of OPA1 totally disrupts mitochondrial integrity, the cells cannot tolerate this state and undergo apoptosis. $OPA1^{-/-}$ mice are the first *in vivo* model that tries to explain the 'mysterious' function of OPA1. As discussed above, it might be that different isoforms and processed forms of OPA1 fulfil different roles in mitochondria. The function of the mostly studied form, isoform 1, has now become clearer. Since this isoform was able to restore many, but not all phenotypic changes in $OPA1^{-/-}$ cells (Figure 24 and 28), the necessity for studying other isoforms became obvious. OPA1 knockout MEFs presented here are the first and only cells available that are totally devoid of OPA1. This makes them an excellent tool to study each OPA1 isoform individually. The expression of individual isoforms on a null background will surely be very useful for resolving the exact function of OPA1.

Studies of $OPA1^{+/-}$ mice did not reveal any phenotypic change in their visual or hearing system. Therefore, the future aim is to determine if different genetic backgrounds, change in environmental conditions (like diet or UV light) or age could trigger adOA.