Université de Montréal

Role of E3-Ligase Parkin in mitochondrial quality control in a cardiotoxicity model to anthracyclines

Par François Brisebois

Sous la tutelle du Dr. Yan Burelle, faculté de pharmacie

Université de Montréal

Mémoire présenté à la faculté des études supérieures et postdoctorales en vue de l'obtention du grade de maîtrise

Résumé

Dues à leur importance croissante dans la dégénérescence musculaire, les mitochondries sont de plus en plus étudiées en relation à diverses myopathies. Leurs mécanismes de contrôle de qualité sont reconnus pour leur rôle important dans la santé mitochondrial. Dans cette étude, nous tentons de déterminer si le déficit de mitophagie chez les souris déficiente du gène Parkin causera une exacerbation des dysfonctions mitochondriales normalement induite par la doxorubicine. Nous avons analysé l'impact de l'ablation de Parkin en réponse à un traitement à la doxorubicine au niveau du fonctionnement cardiaque, des fonctions mitochondriales et de l'enzymologie mitochondriale. Nos résultats démontrent qu'à l'état basal, l'absence de Parkin n'induit pas de pathologie cardiaque mais est associé à des dysfonctions mitochondriales multiples. La doxorubicine induit des dysfonctions respiratoires, du stress oxydant mitochondrial et une susceptibilité à l'ouverture du pore de transition de perméabilité (PTP). Finalement, contrairement à notre hypothèse, l'absence de Parkin n'accentue pas les dysfonctions mitochondriales induites par la doxorubicine et semble même exercer un effet protecteur.

Mots clés : mitochondrie, respiration, ROS, mPTP, muscle cardiaque, autophagie, parkin, mitophagie, doxorubicine.

Summary

Mitochondria are becoming the focus of many studies because of their increasingly important role in cellular damage and related myopathies. Their endogenous quality control mechanisms are recognized for their crucial role in mitochondrial health. In our study, we attempted to determine if the deficit of mitophagy in Parkin deficient mice would cause an exacerbation of mitochondrial dysfunctions usually induced by doxorubicin. We have analyzed the impact of the ablation of Parkin in response to treatment with doxorubicin at the level of cardiac functions, mitochondrial functions as well as mitochondrial enzymology. Our results demonstrated that at baseline, the absence of Parkin didn't induce cardiac pathologies but was associated with many mitochondrial dysfunctions. Doxorubicin induced respiratory dysfunctions, mitochondrial oxidative stress as well as greater susceptibility to permeability transition pore (PTP) opening. Finally, contrary to our hypothesis, the absence of Parkin, didn't exacerbate mitochondrial dysfunctions induced by doxorubicin and seemed to have a protective effect.

Key words: mitochondria, respiration, ROS, mPTP, cardiac muscle, autophagy, parkin, mitophagy, doxorubicin.

TABLE OF CONTENT

Abbreviations

AMPK: Adenosine Monophosphate activated Kinase
ANT: Adenine Nucleotide Translocase
ATG: Autophagy-related Genes
ATP/ADP: Adenosine Tri/Diphoshate
BH: Bax Oligomer
Ca^{2+}: Calcium
CypD: Cyclophilin D
DRP : Dynamin-Related Protein
ER: Endoplasmic Reticulum
ETC: Electron Transport Chain
FADH: Flavin Adenine Dinucleotide
FMN : Flavine mononucleotide
GPx : Glutathione peroxidase
GR : Glutathione Reductase
GSH/GSSG : Reduced/Oxidized glutathione
IMS : Intermembrane space
LAMP : Lysosomal associated membrane protein
LC3: Light-Chain
MFN :Mitofusin
MIM : Mitochondrial inner-membrane
MOM: mitochondrial outer membrane
mtDNA: mitochondrial Deoxyribonucleic Acid
mTOR: mammalian Target Of Rapamycin
NADH: Nicotinamide Adenine DInucleotide
OPA : Optic-atrophy factor-1
OXPHOS : Oxidative phosphorylation
PAS : Phagophore Assembly Site
PRx : Peroxiredoxin
PTP: Permeability Transition Pore
QC : Quality Control
RNS : Reactive Nitrogen Specie
ROS: Reactive Oxygen Species
SOD: Superoxide Dismutase
TOM/TIM: Translocase Outer/Inner Membrane
VDAC: Voltage-Dependant Anion Channel
WT:Wild-type

List of Figures

Foreword

This memoir was done under the supervision of Dr. Yan Burelle who runs a laboratory focused on mitochondrial dysfunctions and their implications in different pathologies. His laboratory has the appropriate equipment to study mitochondrial respiration, reactive oxygen species production, calcium regulation through the permeability transition pore, cell death signaling and other mitochondria related events. This memoir appropriately depicts mitochondrial dysfunctions related to doxorubicin-induced cardiotoxicity.

1 Review of Literature

1.1 Introduction:

Mitochondria are specialized organelles originating from the symbiotic engulfment of aerobic-proteobacteria by pre-eukaryotic cells more than 1.5 billion years ago. Over the course of evolution, mitochondria have evolved as specialized organelles with a plethora of cellular functions. They play a central role in adenosine triphosphate (ATP) production through oxidative phosphorylation (OXPHOS). They actively participate in cellular Ca^{2+} dynamics by their capacity to take up and release Ca^{2+}(Bernardi 2013). They generate metabolic outputs, which can modulate multiple signalling cascades, and nuclear gene expression programs through genetic and epigenetic mechanisms. They constitute one of the main sources of reactive oxygen species (ROS), which can participate in cell signalling or cell dysfunction/death under physiological and pathological conditions respectively (Inoue, Sato et al. 2003). Finally, in response to stress-induced signalling events converging to mitochondria, or of intrinsic dysfunctions within mitochondria caused by acute or chronic pathological conditions, these organelles can trigger apoptotic and necrotic cell death through permeabilization of their double membrane system (Bernardi 2013).

Considering the vital importance of mitochondria in cellular homeostasis, sophisticated mechanisms have evolved to prevent the accumulation of mitochondrial functional abnormalities through the continuous turnover of organelles. This process involves the replacement of mitochondrial biomass through mitochondrial biogenesis, and the balanced degradation of dysfunctional or damaged

organelles through various quality control (QC) mechanisms, which remain poorly understood (Kissova, Deffieu et al. 2004).

In the heart, where mitochondria account for 30-35% of cardiomyocyte volume, abnormalities in one or several facets of mitochondrial function have been involved in a wide variety of cardiac diseases including ischemia-reperfusion injury, overload-induced ventricular remodelling, chronic heart failure, genetic cardiopathies, as well as cardiopathies associated with sepsis or anthracyclin-based chemotherapy (Ballinger 2005). However, despite this knowledge, little is known about the role of mitochondrial QC in disease development in the heart, and the relationship between mitochondrial QC and the appearance of mitochondrial dysfunction.

Recently, our laboratory and others have focused on mitophagy, a QC process whereby mitochondria destined for elimination are sequestered in double membrane autophagosomes, and delivered to lysosomes for subsequent degradation (Kissova, Deffieu et al. 2004). Indeed, while this process is emerging as central for proper cellular homeostasis, its role in the heart remains unclear. More specifically, the role of mitophagy in the development of cardiopathies, and the appearance of mitochondrial dysfunction during disease remains largely unknown.

For this reason, the purpose of this M.Sc. thesis was to determine the potential impact of genetically impairing mitophagy on the development of stress-induced mitochondrial and cardiac dysfunction. For this purpose, we chose to use doxorubicin-induced cardiotoxicity as a model due to the well-established impact of doxorubicin on mitochondrial functions and structure, and the clinical relevance of this model.

Considering the nature of the experimental work, the literature review presented below provides an overview of mitochondrial structure and function, with a discussion on the impact of doxorubicin on various facets of mitochondrial biology in the heart. This is followed by the presentation of essential concepts that pertain to mitochondrial QC, particularly mitophagy, and how these processes may be impacted in the context of doxorubicin-induced mitochondrial and cardiac toxicity.

1.2 The essential of mitochondrial biology: Morphology and functions

1.2.1 Mitochondrial Morphology:

Mitochondria, similar to their bacterial ancestors are composed of two membranes, the outer (MOM) and inner (MIM) membranes, which delineate 3 distinct compartments, the cytoplasm, the inter-membrane space (IMS) and the

mitochondrial matrix (1). The MOM, which is characterized by a smooth appearance when imaged by electron microscopy, separates the organelle from the cytosolic compartment. It is composed of 50% lipids and 50% proteins, some being integral and harbouring trans-membrane domains, and others being peripheral. These proteins are involved in various functions including fatty acid and protein import into mitochondria, exchange of hydrophilic solutes, and creation of docking sites to enable interactions between mitochondria and other organelles(Linden, Nelson et al. 1989).

The MIM, which is characterized by a rough appearance when imaged by electron microscopy, has a much higher protein density. It also has a much larger surface area then the MOM, which allows the formation of multiple folds or invaginations called cristae. These cristae form a microenvironment where electron transport complexes and mobile electron carriers forming the respiratory chain are preferentially concentrated to improve their catalytic efficiency (Frey and Mannella 2000). The MIM is in general very impermeable to proteins, metabolites, and solutes. Transport across the membrane is therefore mediated by a wide variety of specific membrane-spanning carrier proteins including the translocase of the inner membrane (TOM) complex, the adenine nucleotide exchanger (ANT), numerous ion and di- or tri-carboxylate carriers, electron transport chain (ETC) complexes, and the ATP synthase.

Finally, the matrix is the innermost compartment where a large variety of biological processes take place. The matrix contains among other components, mitochondrial DNA, which encodes the RNA's required for the synthesis of some ETC complex subunits, and a wide range of enzymes including those of the Krebs cycle and β-oxidation, which are essential for biosynthetic processes and energy metabolism.

As shown in Figure 1 mitochondria has typically been represented as a bean-shaped organelle. However, it is now commonly accepted that mitochondrial morphology can vary extensively, ranging from this classical bean-shaped appearance to elongated and highly branched network-like configurations. Moreover, experimental evidence revealed that mitochondrial morphology is *i)* dynamic, *ii)* tightly regulated by specific sets of proteins including Mitofusins (MFN1, MFN2), Optic-atrophy factor-1 (OPA1), and Dynamin-Related Protein-1 (DRP1), and *iii)* intimately linked to mitochondrial QC, and key mitochondrial functions such as respiration, ROS production, and signalling of cell death (Inoue, Sato et al. 2003) (Bernardi 2013).

Figure 1: Mitochondrial Structure

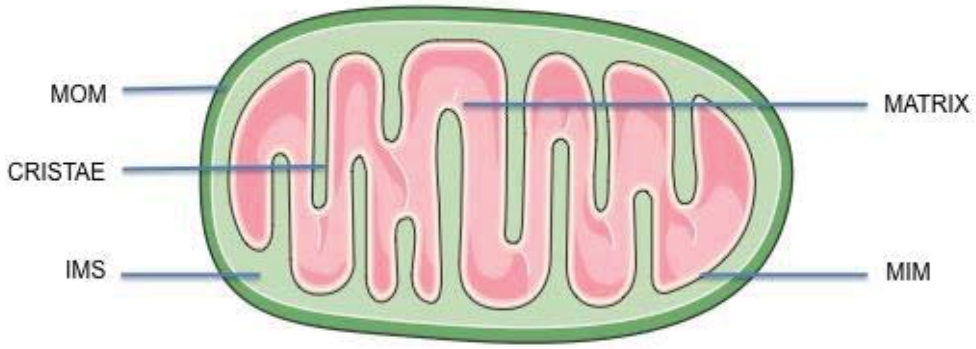

Figure 1: Mitochondrial Structure
MOM: mitochondrial outer-membrane
IMS: intermembrane space
MIM: mitochondrial inner-membrane

1.2.2 Mitochondrial OXPHOS

Mitochondria are central hubs of cellular bioenergetics capable of converting redox energy contained in the C-H bonds of nutrients, into phosphate energy contained in ATP (2). To achieve this function, mitochondria rely not only on its own array of metabolic processes (*e.g.* the Krebs cycle, β-oxidation, and oxidative phosphorylation (OXPHOS) machinery) but also on interactions with other pathways (ex: glycolysis), or organelles (ex: peroxisomes) involved in intermediary metabolism, which provide them with key substrates such as pyruvate and fatty acids, and with reducing equivalents (*e.g.* NADH and $FADH_2$) that can directly fuel OXPHOS for ATP synthesis (2).

The process of OXPHOS relies on the electron transport chain, a series of four multi-units metal-containing complexes (CI, CII, CIII, CIV), and two mobile electron carriers (coenzyme Q and cytochrome c) present in or on the MIM particularly in cristae (2). Together, these proteins are involved in a series of oxido-reduction reactions whereby chemical energy contained in NADH and $FADH_2$ is converted into a proton electrochemical gradient ($\Delta\mu H^+$) across the MIM, which can in turn be used to drive energy consuming processes such as ATP synthesis (2).

More specifically, NADH feeds its electrons at the level of NADH-CoQ dehydrogenase (CI), where electrons are transferred to Coenzyme Q (CoQ) through a

series of oxido-reduction steps involving flavine mononucleotide (FMN) and several iron-sulphur clusters. Reduced CoQ (QH_2), a lipid mobile carrier than shuttles the electrons to CoQ-cytochrome *c* reductase (CIII), where they are transferred to the mobile electron carrier cytochrome *c* through a succession of reaction involving heme and iron-sulfur clusters containing subunits. Reduced cytochrome c ultimately shuttles these electrons to cytochrome c oxidase (CIV), where they are transferred to molecular oxygen through a series of steps involving iron and copper containing subunits, thus allowing the formation of H_2O. During this process, these redox reactions allow CI, CIII and CIV to pump protons across the MIM ($12H^+$: $4H^+$ per complex per mole of NADH) to generate the proton electro-chemical gradient (2).

As for $FADH_2$, the other electron shuttle of importance, it feeds its electrons at the level of succinate dehydrogenase (SDH or CII), which transfers them to the ubiquinone pool directly. In doing so, this electron pathway bypasses CI, thereby reducing the bioenergetics efficiency of $FADH_2$ ($8H^+$/mole of $FADH_2$) compared to NADH ($10H^+$/mole of NADH). Physiologically, the $FADH_2$ and NADH electron pathways function in parallel, although the proportion of NADH *vs* FADH2 may vary depending on the mixture of energy substrate oxidized (2).

Like most metabolic process, the levels of substrates and end-products exert a thermodynamic control on the overall reaction rate of the ETC. This means that the electron flow, and thus oxygen consumption is regulated by the $NADH/NAD^+$

$FADH_2/FAD^+$ (*e.g.* the substrates) ratios on the one hand, and $\Delta\mu H^+$ (the product) on the other hand. Processes that consume $\Delta\mu H^+$, or stimulate the production of NADH and $FADH_2$ will increase flux through the ETC, while high $\Delta\mu H^+$ and more oxidized $NADH/NAD^+$ and $FADH_2/FAD^+$ ratios will lower ETC flux (2).

ATP synthesis is one of the main processes that consume $\Delta\mu H^+$. This is achieved by the FoF1-ATPase located in the MIM, which couples the re-entry of protons into the mitochondrial matrix to the synthesis of ATP from ADP and Pi. In addition, a number of other processes tend to dissipate $\Delta\mu H^+$, and thus stimulate respiration including: *i)* passive or facilitated diffusion of H^+ directly through the MIM, or through uncoupling proteins (UCP's), *ii)* electrogenic transport of several ions and metabolites and *iii)* physiological or pathological slippage of proton pumps along the ETC. *In vivo*, mitochondrial respiration rates will therefore vary considerably depending on ATP demand, but will also be modulated by the various energy consuming or dissipating processes (2).

From a physiopathological standpoint, a number of factors can negatively impact the capacity of mitochondria to generate ATP. This includes shortage of metabolic substrate and/or O_2, dissipation of $\Delta\mu H^+$ independent of ATP synthesis, inhibition of and/or physical damage to one or several complexes involved in OXPHOS, and damage to mtDNA that impair the capacity to synthetize new ETC complexes. As

reviewed below, doxorubicin directly impacts myocardial OXPHOS through several of these mechanisms (2).

Figure 2: The Electron Transport Chain

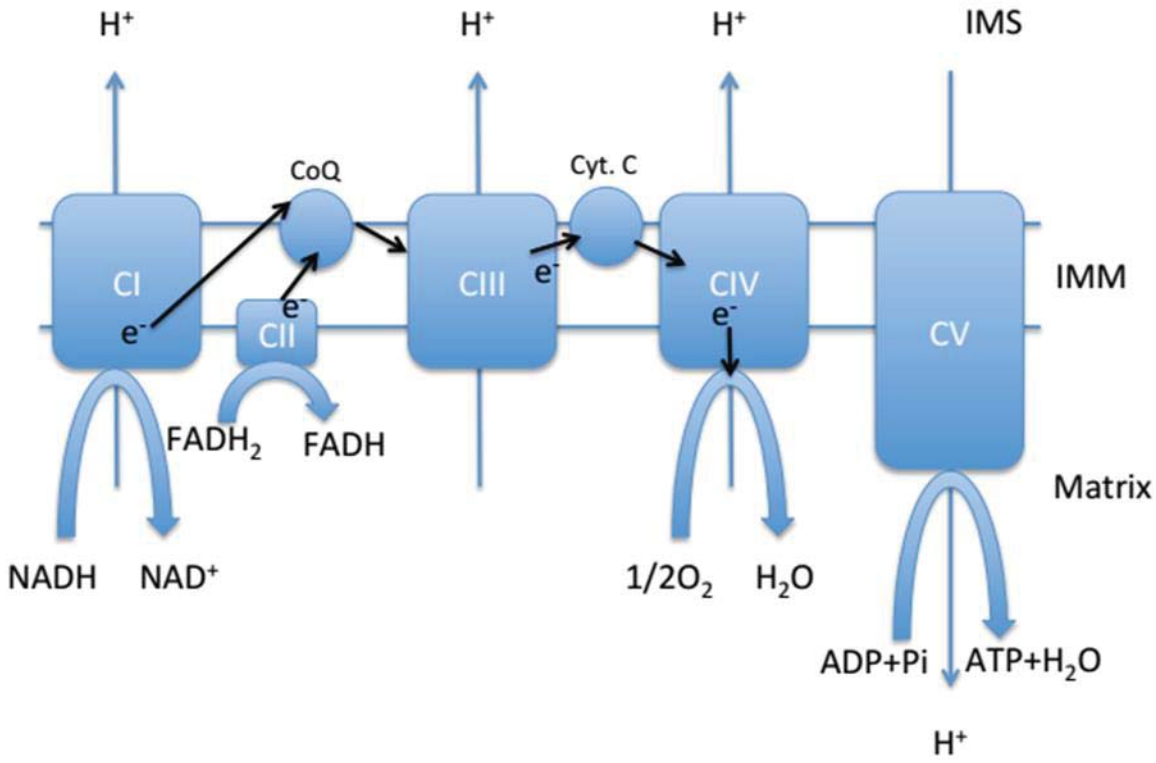

Figure 2: The Electron Transport Chain
By pumping protons into the IMS, the ETC creates an electrochemical gradient across the IMM or MIM enabling re-entering of protons through the complex V and synthesis of ATP.

1.2.3 Reactive oxygen species

In addition to ATP production, mitochondria are also known as important sources of cellular reactive oxygen species (ROS). In general, free radicals are species containing one or more unpaired electrons, conferring them an important degree of reactivity. Oxygen being itself a free radical, it is a favoured recipient of unpaired electrons. Thus, the superoxide anion ($O_2^{-}\bullet$) is almost always the first step in the formation of free radicals and is accordingly referred to as the primary reactive oxygen species (ROS) (Inoue, Sato et al. 2003). $O_2^{-}\bullet$ either interacts with nitric oxide based molecules to form reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^{-}\bullet$), or, is dismutated into the more stable non-radical ROS, hydrogen peroxide (H_2O_2), by superoxide dismutases (SOD). Finally, hydrogen peroxide can lead to the formation of potentially damaging secondary ROS, through enzyme- or metal-catalyzed reactions (Valko, Leibfritz et al. 2007) (3).

Although several sources of $O_2^{-}\bullet$ exist in cells, mitochondria, which harbor in their ETC a complex succession of oxido-reduction reactions, constitute one of the major production sites. Complexes I and III of the ETC are known as the principle contributors to mitochondrial $O_2^{-}\bullet$ production. Historically, complex III in particular was suggested as the main source of superoxide particularly under conditions of high redox states (*i.e.* inhibition of complex III downstream of the Q cycle), which promote the accumulation of ubisemiquinone, the reactive intermediate of the Q cycle (Turrens 2003). Ubisemiquinone then reacts with molecular oxygen to generate $O_2^{-}\bullet$

primarily in the mitochondrial matrix but also in the inter-membrane space (St-Pierre, Buckingham et al. 2002) (Han, Williams et al. 2001) (3).

However, it is increasingly recognized that complex I may actually be the main physiological source of $O_2^{-}\bullet$ in the ETC. Complex I generates superoxide at the level of the flavin mononucleotide centers (FMN) under conditions of forward electron when the complex I is fed with NADH. This is particularly evident when complex I is inhibited downstream of FMN centers by rotenone, which will increase the reduction state of FMN centers and promote electron leaks. In addition, complex I can also generate large amounts of $O_2^{-}\bullet$ when the ETC is fed with substrates for complex II (i.e. with $FADH_2$ donors). In this condition, $FADH_2$ allows to reverse electron flow through complex I, which causes a large increase in $O_2^{-}\bullet$ at the level of the ubiquinone binding site (Q site). This phenonemon is exacerbated under conditions where forward and reverse electron flow are promoted by the simultaneous presence of NADH and $FADH_2$ donors to the ETC, which causes an important reduction of complex I. ROS production in vivo is therefore likely to be very sensitive to changes in redox state and to the absolute electron flux through the ETC (Turrens 2003, Brand 2010) (3).

Being a primary site of potentially damageable $O_2^{-}\bullet$, mitochondria are endowed with a complex array of enzymatic and non-enzymatic antioxidant systems. Manganese-dependent superoxide dismutase (Mg-SOD or SOD_2) is abundently expressed in the mitochondrial matrix where it is responsible for the rapid dismutation of $O_2^{-}\bullet$ into H_2O_2., while Zinc-dependent SOD (Zn-SOD or SOD1), is

responsible for the dismutation of $O_2^{-}\bullet$ in the intermembrane space and cytosol. A number of enzymatic systems exist to eliminate H_2O_2 within mitochondria including i) the glutathione system catalyzed by glutathione peroxidase and reductase, ii) catalase, and iii) the peroxiredoxins system. In addition to these enzymatic systems, non-enzymatic antioxidants such as tocopherol (Vitamin E), retinoids (Vitamin A) or ascorbates (Vitamin C) also confer some degree of protection against oxidative stress (Inoue, Sato et al. 2003) (Cadenas and Davies 2000) (3).

When the balance between oxidants and antioxidants is lost, there is excess in ROS production and subsequent creation of oxidative stress. This can lead to mitochondrial DNA damage as the hydroxyl radical ($OH^{\cdot}$) reacts with both the purine and pyrimidine bases of DNA. By doing so, it permanently damages the genetic material of the cell by modifying its composition. Oxidative stress can also cause peroxidation of membrane phospholipids. The third common side effect of excess ROS is protein damage. The proposed mechanism of action is the formation of a carbon-centered radical from reacting with a hydroxyl radical. The carbon-centered complex creates a peroxyl radical by reacting with oxygen to finally react with a protonated superoxide forming alkyl peroxide. These reactions can ultimately cause peptide bond cleavage. (Valko, Rhodes et al. 2006).

Although ROS/RNS can cause harm, their presence to a certain extent is required for normal physiological function. In fact, physiological ROS production, by activating transcription factors such as MAP-Kinase/AP-1 and NF-kB pathways, is

necessary for intracellular signaling, regulation, proliferation and apoptosis. Preservation of this delicate equilibrium is thus crucial to maintain normal cellular responsivity and avoid adverse effects of oxidative stress on nucleic acids, membrane lipids, and proteins (Devasagayam, Tilak et al. 2004).

In terms of doxorubicin-induced cardiac dysfunction, we will review later that generation of excess $O_2^{-}\bullet$ at the level of the ETC is a central mechanism driving mitochondrial damage and ultimately cardiomyocyte abnormalities.

Figure 3: Mitochondrial ROS production and Scavenging

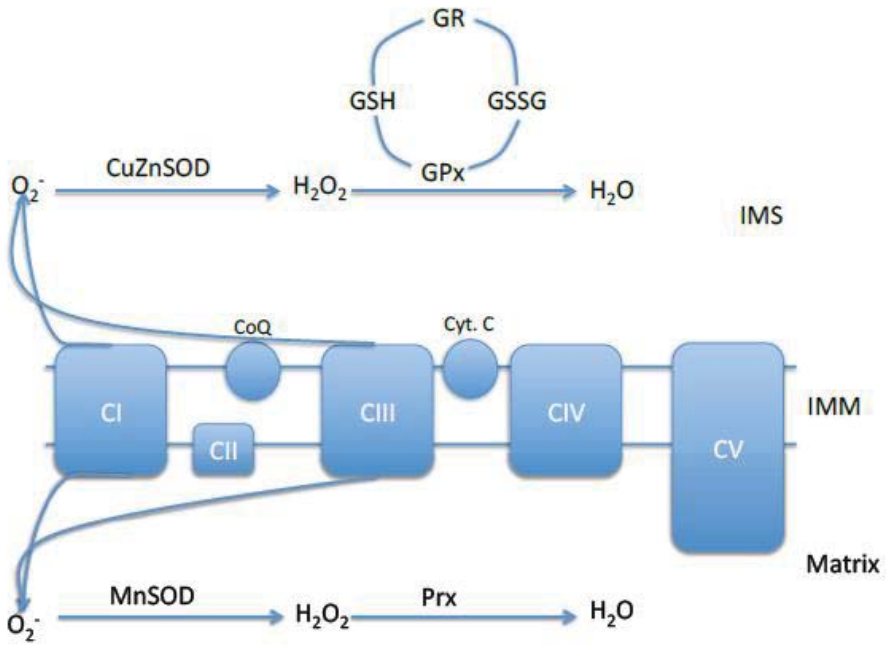

Figure 3: Mitochondrial ROS production and Scavenging
In the cytosol, CuZnSOD detoxifies superoxide anions produced at the complexes I and III of the electron transport chain into hydrogen peroxide before being transformed into water by glutathione peroxidase. Inside the matrix, MnSOD detoxifies the superoxide anions produced by both complexes I and III before peroxiredoxin detoxifies the peroxide into water.
SOD: Superoxide dismutase. Gpx: glutathione peroxidase. Prx: Peroxiredoxin. GR: Glutathione Reductase. GSH/GSSG reduced/oxidized glutathione.

1.2.4 Mitochondrial membrane permeation and cell death signalling

Mitochondria also play a key role in the regulation of cell death by their capacity to trigger necrosis and apoptosis through permeation of their membranes.

Membrane permeation can result from the activation of multiple signaling pathways that converge to mitochondria, or from intrinsic dysfunction within mitochondria caused by acute or chronic pathological conditions (Kroemer, Galluzzi et al. 2007) .

There is emerging evidence that there is an important distinction to be made between outer and inner membrane permeabilization, with each having different modes of regulation and different physiological implications (Kroemer, 2007, Phys Rev). Although the molecular underpinnings differentiating the two remain unclear, the consensus so far establishes that outer membrane permeabilization is performed by the insertion of Bcl-2 family BH-3 oligomers into the outer membrane, while inner membrane permeabilization is achieved through opening of the mitochondrial permeability transition pore (mPTP), which, as described later, is believed to be involved in doxorubicin-induced mitochondrial dysfunction (Chacon and Acosta 1991).

1.2.4.1 The permeability transition pore

Mitochondrial permeability transition was initially described on isolated mitochondria as a sudden increase in membrane permeability to solutes in presence of high Ca^{2+} levels. While this phenomenon was believed to be caused by non-specific membrane damage, it is not widely recognized to be mediated by the mPTP, a high conductance non-specific channel of the inner membrane, which according to the

latest model, is formed by ATP synthase dimers at the crest of mitochondrial cristaes (Bernardi 2013). Prolonged and irreversible opening of this pore allows the equilibration of solutes of < 1500 Da across the mitochondrial membrane, which has several consequences including i) loss of membrane potential, ii) ATP hydrolysis through reversal of the ATP synthase, iii) swelling of the mitochodnrial matrix, and ultimately rupture of the outer mitochondrial membrane, and iv) release of several apoptotic factors including cytochrome c, AIF, SMAC/DIABLO and Endonuclease G (Du, Wang et al. 2004).

Regulation of the mPTP is complex and implies a number of modulators. Accumulation of Ca^{2+} in the matrix is the single most important inducer of mPTP opening (Choo, Johnson et al. 2004). However, several factors facilitate pore opening by increasing its sensitivity to Ca^{2+} accumulation of inorganic phosphate, reduced membrane potential, depletion of ATP and ADP, and oxidative stress being some of the most important ones (Brenner and Moulin 2012). In contrast, divalent cations such as Mg^{2+} and adenylates which all bind to the ATP synthase, act as partial inhibitors (Beutner, Ruck et al. 1998).

In addition to these regulators, cyclophilin-D (Cyp-D), a member of the immunophilin family protein located exclusively in the mitochondrial matrix constitutes an important endogenous sensitizer of the mPTP (Baines, Kaiser et al. 2005). In response to Ca^{2+} overload or oxidative stress, CypD which is soluble in the

matrix is recruited from the inner membrane where it binds the lateral stalk of ATP synthase dimers, thus promoting its conversion into the mPTP configuration (Bernardi 2013). Cyclosporin-A, the prototypical partial inhibitor of mPTP opening was shown to bind Cyp-D specifically thus preventing its membrane recruitment under conditions of mitochondrial stress (Bernardi, Krauskopf et al. 2006).

Although the molecular identity of the mPTP has remained elusive for a long time, recent studies by the group of Bernardi have provided strong evidence that it is in fact formed by ATPsynthase dimers located at the crest on mitochondrial cristae (Bernardi 2013). This model is currently the only one able to account for all the functional and regulatory features of the mPTP that were previously established (Bernardi 2013).

1.3 Mitochondrial quality control

Maintenance of an optimally functioning mitochondrial pool is absolutely essential for cellular homeostasis, particularly in the heart, in which these organelles occupy more than 30 % of cellular volume. Over the recent years, it has become clear that this essential function is achieved through the action of several quality control mechanisms that collectively insure mitochondrial repair, and/or clearance of damaged molecular components or entire organelles. In particular, general autophagy, and the more specific form of autophagy termed mitophagy have emerged as important determinants of mitochondrial health, particularly in cells that are

terminally differentiated and are rich in mitochondria such as cardiomyocytes (Gottlieb, Finley et al. 2009).

In this section, I will begin by depicting a general overview of autophagy and its mechanisms. I will follow this up with a description of a more selective but still misunderstood autophagic process called mitophagy. .

1.3.1 Autophagy: Mechanisms and Pathways

Autophagy consists of a very important and complex cellular process that enables cells to maintain their energy levels in situations of starvation and rid themselves of protein aggregates and damaged organelles. Many molecular pathways allow autophagy to work its course in response to many different situations such as starvation, infections, and damage induced by stress and cellular alterations. There are different subgroups of autophagy depending on the organelles or proteins targeted for degradation. In fact, there is macro-autophagy and micro-autophagy described by De Duve and Wattiaux as well as chaperones mediated autophagy explained by Dice(De Duve and Wattiaux 1966) (Chiang, Terlecky et al. 1989) . In this section of the literature review, we will focus on macro-autophagy (autophagy).

Regulated by the insulin pathway, starvation is an extensively researched method of promoting autophagy. Conversely, a high caloric diet can inhibit

autophagy, thus playing a role in obesity. Type II diabetes, sedentary lifestyles, and metabolic syndromes are also characterized as having reduced levels of autophagy (Donati 2006) (Etgen, Oldham et al. 2002). An experiment in which a common autophagic regulator, p62 was knocked out resulted in insulin resistant obese mice proving the clear relationship between autophagy and metabolism (Rodriguez, Duran et al. 2006). There are many other examples of the protective virtue of autophagy. Amongst these, in an acutely induced stressful environment such as ischemia-reperfusion, murine myocytes have been found to have a highly autophagic response (Gustafsson and Gottlieb 2008). Exercise has been known to be cardioprotective for quite some time, but is now also confirmed to be correlated with a high autophagic response (Dohm, Tapscott et al. 1987). Autophagy has also been shown to be related to ageing since many autophagic genes such as Atg2, Atg8a, Atg18, and bchs exhibit decline in expression with times allowing for subsequent higher expression of dysfunctional protein aggregates (Simonsen, Cumming et al. 2008).

To paint a big picture, the autophagic cargo is engulfed by a double-membrane structure built from nearby organelle membranes (autophagosome) and fuses with lysosomes for degradation purposes enabled by many lysosomial enzymes. Work in yeast resulted in the discovery of 35 Autophagy Related Genes (Atgs), which are highly conserved proteins that play important roles in the molecular pathways of autophagy (Takeshige, Baba et al. 1992) (Tsukada and Ohsumi 1993) (4). There are 4 main steps in the autophagic process. The first step consists of the initiation of the

formation of phagophore assembly site (PAS: Phagophore Assembly Site). The second step is the elongation and closing of the autophagosome. The third step is the maturation of the autophagosome by fusing with the lysosome. Finally, the fourth step consists of the degradation and recycling of the autophagic cargo by lysosomial enzymes. Throughout these 4 steps, complex signalization and regulatory stimuli act at different levels (4).

The initiation of the formation of PAS is crucial since it establishes what cellular organelle is to be degraded. Ubiquitine ligase enables ubiquitination of proteins. This allows the recruitement of P62-SQSTM1, which interacts with the ubiquitin and allows the damaged proteins destined for degradation to link to an LC3 protein (homologue to ATG8), which is found at the surface of PAS (Pankiv, Clausen et al. 2007) (Itakura and Mizushima 2011) (Kabeya, Mizushima et al. 2000).

Once the damaged organelle is recognized, membrane precursors initiate the formation of the double membrane of the autophagosome near the damaged material destined for degradation. Autophagosomes can originate from different sources such as the endoplasmic reticulum, the Golgi apparatus, the endosomes, the plasmic membrane and even the mitochondrial membranes (Hayashi-Nishino, Fujita et al. 2009).

In mitochondria, formation of the autophagosomes has been found to be dependent on mitofusine 2 (MFN2), which is a protein that also plays an important role in fusion and fission of the mitochondrial network. This points to a clear relationship between regulation of autophagy and mitochondrial morphology remodeling (Zhao, Huang et al. 2012).

In addition to the linking of the damaged organelles to the autophasome, the formation, elongation and closing of the later requires the assembly of a series of proteins (Atgs) at the PAS. These Atgs belong to two conjugation systems: Atg5,12,10 and Atg4,3,7(Suzuki, Kubota et al. 2007). Together, these systems enable the formation of molecular complex on Atg16L and the subsequent insertion of LC3 in the membrane of PAS (Kang, Zeh et al. 2011).

As mentioned earlier, the autophagy machinery is the result of many different complex signalization pathways. The most characterized of these is focuses on mTOR (Mamalian Target of Rapamycin). MTOR is a kinase that plays a crucial role in the coupling between the energetic cellular stage and the anabolic/catabolic balance. Although both mTORC1 and mTORC2 exists, only mTORC1 is sensitive to rapamycine and plays a role in autophagy. As an important metabolic sensor, activated mTOR prevents autophagy to rather favor protein synthesis. However, in a situation of starvation and energy deficit, inhibition of mTORC1 leads to autophagy signalization in coordination with other signaling pathways such as FOXO3a-NFkB, which

regulates transcription of autophagic regulated genes. The mTOR complex goes on to link itself to Unc-51-like kinase1 (ULK1) and Atg proteins enabling initiation and elongation before disassociating itself to bind to the endoplasmic reticulum (ER) (Laplante and Sabatini 2012). Inactivation of mTORC1, in state of starvation prevents the phosphorylation of mATG13, thus enabling formation of complex ULK1- mATG13-mATG17 (Lv, Huang et al. 2014). This shows the critical role of mTOR as a regulator in the autophagy process as a whole.

The specific orientation and localization in the intracellular space of mTORC1 is necessary and enabled by phosphatidylinositol-3-phosphate (PI(3)P) at the membranes. Pi(3)P formation results from PI3Kinase-III Vps34, which is recruited at PAS due to ULK1 Beclin-1 complex, which itself is recruited to the ER. The Beclin1 Vps34 complex is thus crucial in the process resulting in elongation of the phagophore.

After the phagophore has completed the elongation process, its membrane extremities fuse together enabling it to close and become an autophagosome. During this closing process, the phagophore is slightly modified to allow its fusion with the lysosome once it has become an autophagosome. One of the most important modifications stated above consists of the cleavage of LC3 proteins of the outside membrane of the autophagosome by ATG4 (Kirisako, Ichimura et al. 2000).

There are many proteins that play the role of fixation adaptors to the membrane and facilitate the fusion of the autophagosome to the lysosome. For example, Rab7-interacting lysosomal protein (RILP) causes RAB7 to allow for the formation of tubules between the autophagosome and the lysosome, while FYCO1 activates RAB7 enabling the transfer of the autophagosome to the lysosome along these tubules (Harrison, Bucci et al. 2003). Another example of fixation adaptor protein is TECPR1, which enables the elongation process by allowing the interaction between ATG5-ATG12 and PI3P (Chen, Fan et al. 2012).

Another important group of proteins that play an essential role in the fusion process are lysosomal-associated membrane proteins LAMP1 and LAMP2. In fact these proteins are adaptors at the interface between the lysosome and autophagosome. Interestingly, animal KO studies have only shown a strong autophagosome accumulation in LAMP2 KO (Jager, Bucci et al. 2004).

After the autophagosome has completely fused to the lysosome, the damaged organelles are degraded by many hydrolases, lysosomial proteases as well as the acidic environment found in these organelles.

While autophagy is necessary to ride the cell of its damaged organelles, excess autophagy is equally detrimental. For this reason, there are many mechanisms of autophagy inhibition although most are still misunderstood.

An important mechanism of autophagy inhibition is sequestration of BECLIN-1 at the endoplasmic reticulum surface by BCL2 proteins such as BCL2 and BCL-Xl, which are more commonly known for their anti-apoptotic behavior (Bonnefoy-Berard, Aouacheria et al. 2004). While BCLs can inhibit autophagy by linking itself and sequestering Beclin, it is unable to do so in a situation of cellular stress or lack of energy. It was also recently discovered that the interaction between BCL2 and BECLIN-1 requires nutrient-activated autophagy factor-1(NAF-1). In fact, studies showed ablation of NAF-1 prevents BCL-2 from linking to BECLIN-1, thus stimulating autophagy (Chang, Nguyen et al. 2010).

There are many other mechanisms by which autophagy can be inhibited at all different steps of the autophagic process. While MTOR can inhibit autophagy at the induction stage, PI(3)P dephosphorylation, BCL-2 and NAF-1 prevent formation of the previously mentioned PAS. Autophagy can also be inhibited at the elongation process. For instance, the FLIP protein limits lipidation of LC3, preventing the elongation process to take place (Khalfan and Klionsky 2002).

Although its exact mechanisms are still unknown, autophagy has been shown to play a major role in cardioprotection by disabling damaged proteins to overly harm their cellular environment (Cuervo, Bergamini et al. 2005). Lysosomes have been shown to be critical for adequate heart functioning. In fact, deficiencies in lysosomal

degradation capacities resulted in dilated cardiomyopathies. Defects related to mutations of lysosomal-associated membrane proteins have also been shown to cause acute cardiomyopathies just like in Danon disease (Axe, Walker et al. 2008) (Itakura and Mizushima 2010) (Klionsky, Baehrecke et al. 2011).

The cardioprotective role of autophagy has also been studied in pre-conditioning situations. Many of the agents used to mimic pharmacological conditioning are potent sources of autophagy. In chronically ischemic environments, autophagy has been found to be upregulated and cardioprotective (Gustafsson and Gottlieb 2008).

Figure 4: Macro-Autophagy pathways

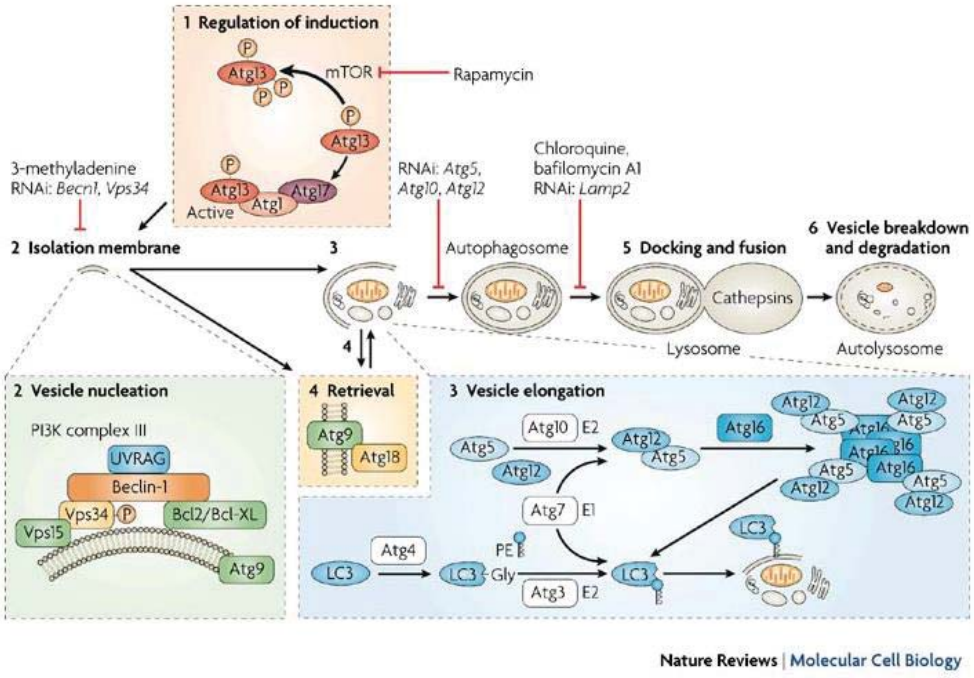

Figure 4: Macro-Autophagy pathways
Here is a depiction of macro-autophagy and its pathways. Image taken from Maiuri et al. 2007. Steps 1 is represented by stages 1 and 2; step 2: 3,4; step 3:5 and step 4:6.

1.3.2 Mitophagy

While autophagy can be non-selective, it can also target specific organelles that are dysfunctional in order to selectively degrade them. Since mitochondria have such a pivotal role in pathologies, it isn't surprising that they have their own quality control mechanism. In this section, we will elaborate on the particular mechanisms behind the specific degradation of damaged mitochondria.

Autophagy of mitochondria has been termed mitophagy. Although healthy mitochondria (efficiently produce ATP, have respectable membrane potential, don't produce much ROS, have few modified proteins, and have good amounts of OPA-1), undergo fusion and fission cycles, damaged mitochondria are excluded from the cycle and are discarded by autophagy. While mitophagy was first discovered in yeast, its mechanisms are fairly misunderstood. One study determined that the mitochondrial protein Uth1p played a role in mitophagy since mitochondrial degradation was delayed in its absence while the autophagic machinery remained active (Kissova, Deffieu et al.2004). Other studies in yeast showed that regulatory intermediates such as Atg32 must also play a role in mitophagy since its mutant cell counterpart prevents mitophagy (Kanki, Wang et al.2009) (Okamoto, Kondo-Okamoto et al. 2009).

Experiments have shown that photodamaged mitochondria were selectively captured by autophagosomes. Membrane potential is important for the importation of mitochondrial proteins. It is thus necessary to have high enough membrane potential to replace damaged proteins (Kubli and Gustafsson 2012).

1.3.2.1 Role of Parkin in mitophagy

In mammals, there are two distinct pathways leading to mitophagy and both of these pathways are activated by the depolarization of the mitochondrial membrane. The first pathway requires adaptors at the mitochondrial membrane, while the second pathway simply relies on receptors of autophagy. Inhibition of complex 1 of the ETC by a meperidine derivative (MPTP) caused Parkinsonian

syndrome (Langston, Ballard et al. 1983). After similar symptoms were discovered in models without the Park 2 gene, a clear relation was discovered between Parkin and mitochondria (Greene, Whitworth et al. 2003). Coded by Park2, Parkin is an E3-ligase that was then studied in mammals because it's mutation in humans were associated with early onset familial forms of Parkinson's disease (i.e 10% of all Parkinson's cases) (Greene, Whitworth et al. 2003).

It is a protein ligase that consists of a 60 amino acid linker surrounded by an N-terminal ubiquitin-like domain and four zinc-finger domains. Although its role is misunderstood, Parkin is mostly studied for its impact on bioenergetics and mitochondrial quality control (Exner, Lutz et al. 2012). The relation between Parkin and mitochondrial maintenance was introduced by loss of function mutations in Drosophila melanogaster (Greene, Whitworth et al. 2003). In fact, Parkin gets recruited to depolarized mitochondria to engage its elimination by specialized autophagy (mitophagy) (Geisler, Holmstrom et al. 2010, Narendra, Kane et al. 2010) (Vives-Bauza, de Vries et al. 2010). Narendra et al. showed that overexpression of Parkin used mitophagy to remove mitochondria that had lost their membrane potential (Narendra, Jin et al. 2010). Since Parkin selectively binds itself to damaged mitochondria, it is believed to be a key player of mitophagy (Grenier, McLelland et al. 2013).

Upon studying Parkin in mammals, the protein was found to be located in the cytosol but recruited to the mitochondria to lead to its degradation. In order to prove that Parkin's autophagic role was specific to the mitochondria, similar in vitro assays were conducted in cells, in which the initiation step of autophagy was inhibited (ATG5 KO). This study showed that Parkin is in fact enabling autophagy specifically at the mitochondrial level (Scarffe, Stevens et al. 2014).

To better understand Parkin's role in mitophagy, one must understand its interaction with Pink1 as depicted in figure 5. Pink1 consists of a mitochondrial serine/threonine-protein kinase that is also mutated in recessive cases of PD (Valente, Salvi et al. 2004). Its role is to protect cells from mitochondrial damage. Pink1 is and upstream regulator of Parkin function since overexpression of Parkin can salvage the phenotype of Pink1 knockout whereas overexpression of Pink1 is incapable of salvaging the absence of Parkin (Clark, Dodson et al. 2006). Many other studies have showed that Pink1 must be present in order for Parkin to be recruited to the damaged mitochondria (Geisler, Holmstrom et al. 2010) (Narendra, Kane et al. 2010) (Vives-Bauza, de Vries et al. 2010).

The Pink1/Parkin pathway depends on the state of the mitochondrial membrane potential. In normal mitochondria, Pink1 makes its way to the inner membrane by the TIM/TOM complex and is cleaved and degraded. However, in mitochondria without membrane potential, Pink1 positions itself on the outer

membrane of the mitochondria and recruits Parkin (Narendra, Tanaka et al. 2008, Narendra, Jin et al. 2010) (Lazarou, Jin et al. 2012). The Pink1/Parkin complex initiates autophagic degradation of the peroxisome specific to the mitochondria (mitophagy) by ubiquitilating outer mitochondrial membrane (OMM) proteins that go on to recruit other proteins to initiate mitophagy (Chan, Salazar et al. 2011) (Geisler, Holmstrom et al. 2010) (Lee, Nagano et al. 2010) (Narendra, Jin et al. 2010) (Tanaka 2010) (Yoshii, Kishi et al. 2011).

In studies where Pink1 was mutated, Parkin wasn't recruited (Geisler, Holmstrom et al. 2010) (Okatsu, Saisho et al. 2010) (Vives-Bauza, de Vries et al. 2010). The detail mechanisms behind the recruitement of Parkin by Pink1 aren't understood. Some suggests a physical interaction between Pink1 and Parkin by in vitro co-immunoprecipitation (Sha, Chin et al. 2010). The nature of the substrates that are polyubiqitinated by Parkin is also unknown. Some of these have been identified such as VDAC, Mfn 1/2, and Bcl-2**.** (Geisler, Holmstrom et al. 2010) (Narendra, Jin et al. 2010) (Gegg, Cooper et al. 2010) (Chen, Gao et al. 2010)**.**

Once Parkin transports dysfunctional mitochondria to the perinuclear region, p62 colocalizes to the mitochondria and enables autophagy by attaching to LC3 for regular autophagic degradation (Okatsu, Saisho et al. 2010).

However, research has shown that p62 is dispensable leading to believe in alternative pathways such as Nix/BNIP3L and BNIP3 (Hanna, Quinsay et al. 2012). When the mitochondrial membrane is depolarized, BNIP3 and NIX, proteins with a BH3 profile like the BCL-2 family of proteins, are recruited to the mitochondrial membrane (Chen, Ray et al. 1997). These proteins are monitored by HIF-1 (cellular hypoxia sensor) and FOXO3 in starved cells (Zhang, Bosch-Marce et al. 2008) (Mammucari, Milan et al. 2007). Upon membrane depolarization, NIX and BNIP3 recruit LC3, which is stabilized by LIR. FUNDC1 is also a mitochondrial protein activated by hypoxia and capable of recuiting LC3II (Liu, Feng et al. 2012).

Mitophagy is also thought to be enabled by cardiolipins. These phospholipids of the inner mitochondrial membrane move to the outer membrane following a depolarization and directly recruits LC3-II. It is important to note however that cardiolipin induced mitophagy seems to result more from dysfunctions in the complexes of the respiratory chain and mitochondrial integrity than depolarization itself (Chu, Ji et al. 2013).

Mitophagy consists of a system of degradation specific to the mitochondria and can result from different situations. In fact, mitophagy can come as a consequence of oxidative stress from hypoxia, starvation or mitochondrial dysfunctions.

Figure 5: Mitophagy and the Pink1/Parkin pathway

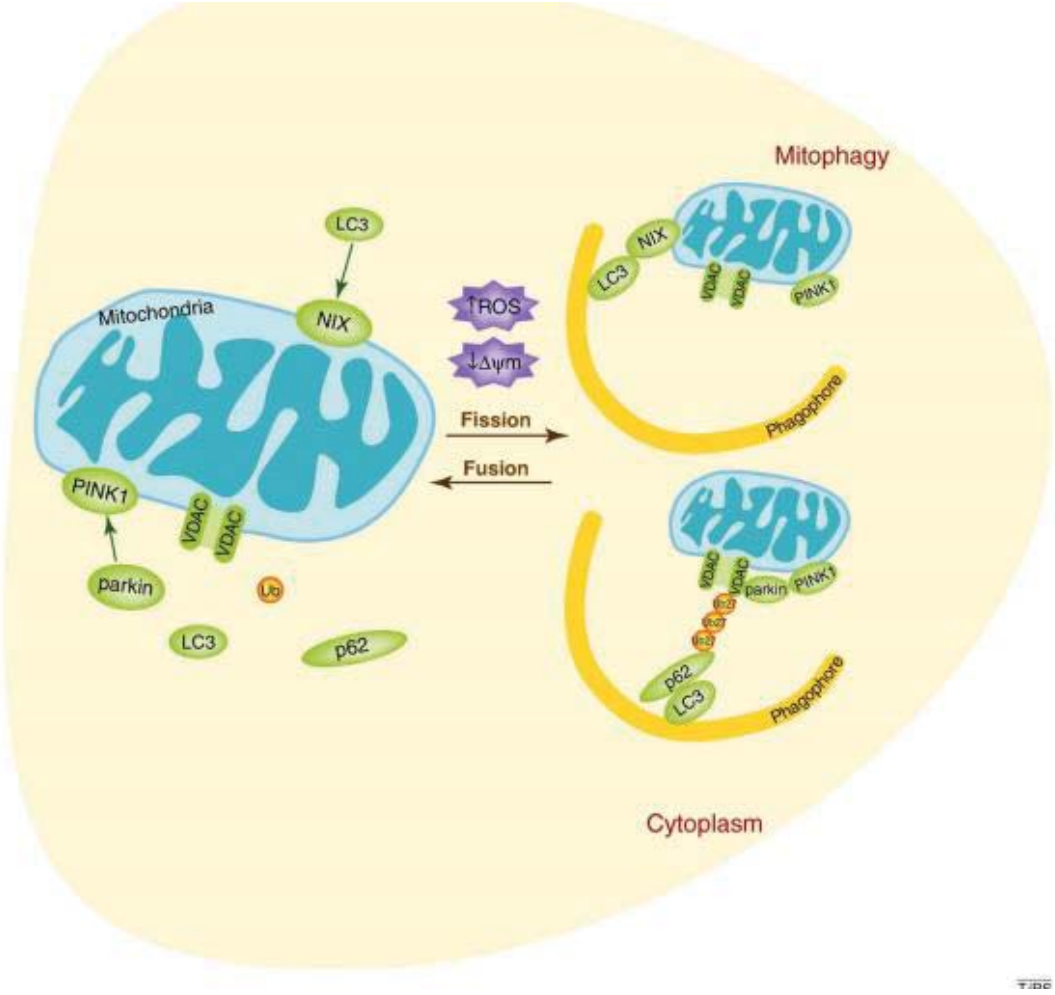

Figure 5: Mitophagy and the Pink1/Parkin pathway
Here is a brief depiction of mitophagy and its pathways. The image was taken from Scherz-Shouval et al. 2011.

1.4 Mitochondrial dysfunctions in cardiotoxicity model to anthracyclines

1.4.1 Doxorubicin short term-cardiotoxicity and long term heart failure

Cancer is characterized by uncontrolled cellular proliferation resulting in the formation of tumors. The general objective of anti-cancer therapies, is to interfere with key processes involved in cellular proliferation, in order to promote commitment of cancer cells to apoptotic or necrotic cell death or senescence. However, due to the relative inefficacy in targeting anti-cancer drugs specifically to tumors, most chemotherapeutic treatment induce collateral damage in healthy tissues and organs.

Doxorubicin is a common chemotherapy compound used for the treatment of several cancers including lymphoblastic and myeloblastic leukaemias, neuroblastomas, bone marrow sarcomas, malignant lymphomas, and carcinomas of the thyroid, breast, and bladder cancer. Doxorubicin is a member of the anthracyclines, family of natural antibiotics which display a planar ring structure that facilitates its integration in the DNA double helix resulting in the inhibition of DNA replication and transcription in dividing cells (Gewirtz 1999).

Due to its relatively short half-life doxorubicine is preferably administered intravenously. In human, the treatment is usually given over a 3-4 week interval and does not exceed a cumulative dose of 450-550 mg/m^2 because of the exponentially

proportional risk of heart failure (Swain, Whaley et al. 2003). Indeed, acute cardiac failure occurs in about 20% of cases, for patients that receive doses exceeding 500 mg/m^2 (Swain, Whaley et al. 2003). However, at lower doses, cardiac dysfunctions can occur at later stages of the treatment or even up to 20 years post treatment depending on the patient. Strategic dosage control has actually attenuated doxorubicine cardiotoxicity. However, long-term complications are common and drug interaction can also accentuate damages. Currently, the lack of understanding of the pathogenic mechanisms underlying antracycline-induced cardiotoxocity remains an obstacle to the development of cardioprotective countermeasures (Swain, Whaley et al. 2003) (Zhang, El-Sikhry et al. 2009).

Rodent models generally recapitulate key features of the cardiac disease observed in patients following both acute and chronic treatment. For instance, in mice, left ventricular pressure is significantly reduced following acute treatment with 15mg/kg/day of doxorubicin for 3 days (Zhang, Shi et al. 2009). An increase in telesystolic diameter is also observed following chronic treatment with 1.5 or 3 mg/kg/week doxorubicin during 5 weeks followed by a 2 week wash-out period (Zhang, Shi et al. 2009). The availability of these models allowed to investigate the cellular mechanisms underlying cardiac toxicity.

1.4.2 Impact of Doxorubicin on mitochondria

Among the mechanisms identified, mitochondrial dysfunctions are suggested as one of the major contributor. Indeed, several studies have shown that Doxorubicin accumulates in mitochondria where it promotes oxidative stress, which in turn induces several deleterious consequences such as: oxidative damage to proteins, lipids, and mtDNA, reduction of ETC and Krebs cycle enzymes activity, respiratory impairments, increase propensity to opening of the PTP, and activation of mitochondria-dependent cell death signalling (Yen, Oberley et al. 1999) (Santos, Moreno et al. 2002) (Serrano, Palmeira et al. 1999).

1.4.2.1 Oxidative stress

Excessive generation of ROS is a critical factor underlying the cardiotoxic effect of doxorubicin. Initially, supports for this hypothesis came from studies showing that, 5-Iminodaunorubicin, which does not lead to excessive ROS production had no adverse effect on mitochondrial and cardiac functions (Davies and Doroshow 1986). Further mechanistic studies revealed that doxorubicin entertains a futile redox cycle at the level of complex 1 of the respiratory chain, where it allows production of semiquinone intermediates (Davies and Doroshow 1986), which are oxidized to form highly reactive superoxide anions that overwhelm mitochondrial antioxidant capacities.

This effect of doxorubicin is facilitated by the fact that this drug displays a particular affinity for mitochondrial lipids, particularly cardiolipin. Owing to this affinity, doxorubicin readily insert itself in the mitochondrial membranes, where it promotes lipid peroxidation (Huart, Brasseur et al. 1984). Malondialdehyde (MDA) levels, a known marker of lipid peroxidation, are increased by nearly threefold in heart mitochondria from rats treated with 6 injections of 2.5mg/kg i.p. of doxorubicin over a 2 week period (Arafa, Mohammad et al. 2014). The accumulation of oxidative stress is also evident in mitochondrial DNA in comparison to the nuclear DNA. Indeed, after acute doxorubicin intoxication, studies revealed a 2-fold increase in 8-hydroxy-2'deoxyguanosine (8OHdG) adducts, a marker of DNA oxidative damage, in mitochondrial DNA relative to nuclear DNA, which clearly support preferential oxidative damage to mitochondria. Studies also show that mitochondrial dysfunctions and oxidative damage to mtDNA persists five weeks after an 8 week treatment with doxorubicin (Serrano, Palmeira et al. 1999), illustrating the fact that damage incurred at the level of the electron transport chain, particularly complex I enables a self-perpetuating cycle of oxidative stress weeks following the last injection.

1.4.2.2 Cellular respiration impairments caused by oxidative stress (ETC, Kreb cycle enzymes and respiratory impairments)

As mentioned above, doxorubicin-induced oxidative stress can lead to the inhibition of ETC and Krebs cycle enzymes, ultimately resulting in respiratory

impairments. For instance, exposure of isolated mitochondria to doxorubicin was shown to lead to a 35% decrease in complex I activity (4.5 ± 0.6 *vs* 7.0 ± 0.9 nmol/min/mg) (Santos, Moreno et al. 2002) resulting in a 20 % decrease in ADP-stimulated state 3 respiration (336 ± 26 *vs* 425 ± 53 natoms O/min/mg), but no changes in basal state 4 respiration in presence of substrates for complex I. Similarly Solem et al. reported a significant reduction of state 3 respiration (358 ± 81 *vs* 529 ± 48 natoms O/min/mg protein), reduced respiratory control ratio (RCR) (5.2 ± 1.3 *vs* 9.3 ± 0.6) and no change in basal complex I-driven state 4 respiration following treatment with doxorubicin (Solem, Henry et al. 1994). Another study in mice treated with doxorubicin reported similar observation. However, this study also reported that doxorubicin led to reduced complex II activity and thus impaired state 3 respiration and RCR in presence of the complex II substrate succinate (Yen, Oberley et al. 1999).

Doxorubicin treatment was also shown to inhibit respiratory chain activity even more severely when coupled in an iron complex. Indeed, in a study performed on pig-heart submitochondrial particles, respiratory chain activity was severely inhibited following administration of an adryamicyn-Iron complex as compared to 10-15% when doxorubicin was administered alone (Demant and Jensen 1983).

Studies have also showed a clear correlation between the extent of lipid peroxidation and respiratory chain inhibition reinforcing the impact of mitochondrial

oxidative stress in doxorubicin-induced respiratory impairments (Demant and Jenson 1983 & Praet et al, 1984).

1.4.2.3 Mitochondrial permeability transition

In addition to the above-mentioned impairments, histopathological analyses have shown that acute and chronic treatments with doxorubicin leads to significant disorganization of mitochondrial structure, and an abnormal swelling of the matrix. (Zhou, Palmeira et al. 2001), which is a typical consequence of mitochondrial membrane permeabilization following opening of the PTP. Furthermore, electron microscopy studies showed that doxorubicin treatment leads to the formation of calcium hydroxyapatite deposits in cardiomyocytes, which localize to mitochondria (Aversano and Boor 1983), further pointing to calcium overload as a trigger for PTP opening. Direct measurement of susceptibility to Ca^{2+}-induced opening of the PTP in isolated mitochondria have repeatedly shown that doxorubicin treatment significantly increases susceptibility to PTP opening (Zhou, Starkov et al. 2001). This susceptibility has been ascribed to the well-established role of oxidative stress in sensitizing the PTP to Ca^{2+} (Chacon and Acosta 1991) (Zoratti and Szabo 1995).

Studies with cyclosporine-A, the prototypical partial inhibitor of the PTP, suggest that opening of the PTP plays an important role in doxorubicin-induced cardiotoxicity. Indeed, administration of cyclosporine-A to doxorubicin-treated rats

prevent the reduction of Ca^{2+}-retention capacity, an indicator of the propensity to PTP opening. Cyclosporin-A was also shown to blunt the sensitization to Ca^{2+}-induced mitochondrial depolarization observed following treatment with doxorubicin (Solem, Henry et al. 1994) (Solem and Wallace 1993).

Overall, it is therefore clear that doxorubicin induces multiple mitochondrial abnormalities encompassing oxidative damage to lipids, proteins and DNA, which ultimately impairs bioenergetics function and promotes triggering of cell death through opening of the PTP. Consequently, mitochondria are increasingly viewed as targets for interventions aimed to limit the cardiotoxic effects of antracyclin-based chemotherapy. Currently proposed strategies include mitigation of oxidative stress using mitochondria-targeted anti-oxidants and prevention of PTP opening using cyclosporine-A derivatives. Furthermore, with the recent discovery of mitochondrial quality control mechanisms such as mitophagy, targeting of these processes is emerging as a future therapeutic avenue to limit mitochondrial dysfunction. However, the role of mitophagy in the heart remains largely unexplored and it is currently unclear whether modulation of this process can have an impact on disease trajectory.

2 RESEARCH HYPOTHESIS AND OBJECTIVES:

My research hypothesis consists that the deficit of mitophagy in Parkin -/- would cause an exacerbation of mitochondrial dysfunctions normally induced by doxorubicin. In order to verify this hypothesis, we submitted Parkin deficient mice to an acute treatment of doxorubicin and analyzed the impact of genotype and treatment on morphology and cardiac functions, diverse mitochondrial functions as well as mitochondrial content and enzymatic activity.

3 EXPERIMENTAL STUDY

3.1 Methodology

3.1.1 Animals

The animal ethics committee of the University of Montreal in conjunction with the Canadian Council of Animal Care approved all the experimental protocols used. Wild type (WT) and Parkin-deficient (Parkin-/-) mice from INSERM-Sanofis-Adventis(Chen, Xu et al. 2011) were taken care of and kept at the Institut de Recherche en Immunologie et en Cancérologie (IRIC) of the University of Montreal. The precise n number used for each experiment is indicated in the figure legend. Mice ranging eight to twelve weeks old were injected i.p with doxorubicin (15mg/kg) or with PBS (vehicle) because these doses had been shown to cause mitochondrial toxicity (Palmeira, Serrano et al. 1997). Injections were made at 0 and 48 hours to emulate the injection timing used in this acute setting (Kawaguchi, Takemera et al. 2012). Animals were then used for experiments five days following the last injection to see if Parkin might have a protective property as established previously in the lab (Piquereau, Godin et al. 2013). Body weight was monitored daily. Animals were then anaesthetized for echocardiography and euthanized by cervical dislocation. Hearts, lungs and livers were rapidly excised and weighed. Subsequently, hearts were either flash frozen and stored in a -80°C freezer or immediately used for the preparations of permeabilized cardiac fibers.

3.1.2 Echocardiography

Mice were sent to the Lady Davis institute for a week for acclimation to their new environment. The following week, mice were subjected to the Doxorubicin treatment protocol as described above. Cardiac morphology and functions were analyzed in anesthetized mice by echocardiography

3.1.3 Permeabilized heart fibers

Left ventricles were removed and placed into pre-cooled buffer A (inmM: (2.77 CaK2EGTA, 7.23 K2EGTA, 6.56 MgCl2, 0.5 dithiothreitol (DTT), 50K-MES, 20 imidazol, 20 taurine, 5.3 Na2ATP, 15 phosphocreatine, pH7.3 at 4°C). Small fiber bundles of the left ventricule were manually dissected under a microscope and permeabilized with saponin (50ug/ml) for 30 minutes. Fiber bundles were then washed three times for 10 minutes in buffer B (in mM:2.77 CaK2EGTA, 7.23 K2EGTA, 1.38 MgCl2, 3.0 K2HPO4, 0.5 dithiothreitol, 20 imidazole, 100 K-MES, 20 taurine, pH 7.3 at 4°C) supplemented with BSA (2 mg/ml) and kept on ice until respirometry is performed and analyzed. In terms of the ghost fibers without myosin, they were permeabilized with saponin and washed three times in Buffer B. They were then washed three times for 10 minutes in Buffer C (in mM: K-MES 80, HEPES 50, taurine 20, DTT 0.5, MgCl2 10, pH 7.3 at 4°C) and incubated for 30 minutes with agitation at 4°C in Buffer D (in mM: KCL 800, HEPES 50, taurine 20, DTT 0.5, MfCl2 10, ATP 10, pH 7.3 at 4°C) to extract myosin. The bundles were finally washed three times in low-EGTA sucrose

buffer (in mM: 250 sucrose, 10 Tris base, and 0.1 EGTA, pH 7.4) and kept on ice until Ca^{2+}-induces PTP assays (Picard, Csukly et al. 2008).

3.1.4 Mitochondrial respiration

Fibers (about 0.3mg dry) bathed in specify volume solution B (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA (100nM free Ca^{2+}), 6.56 $MgCl_2$ (1mM free Mg^{2+}), 20 taurine, 0.5 DTT, 50 K-methane sulfonate (160mM ionic strength), 20 imidazole, pH 7.1)) were used to determine mitochondrial respiratory function. Respiration was measured using Clark type electrodes at 25 ^{o}C under continuous low speed magnetic stirring (5% of maximal speed). Respiratory rates were measured at each step of the following sequence of substrates and inhibitors: the complex I substrates glutamate-malate (GM)(5, 2.5mM); ADP (2mM); complex I inhibitor amytal (2mM); the complex II substrate succinate (5mM); the uncoupler CCCP (1μM); the complex III inhibitor antimycin-A (AA) (8μM); the complex IV substrates N, N', N'- tetramethyl-p-phenylenediamine dihydrochloride (TMPD)-ascorbate (0.9mM, 9mM); and the complex IV KCN (0.6mM). This sequence allowed determining the respiratory performance of mitochondria using specific segments of the electron transport chain as well as various respiratory states including baseline, maximal ADP-stimulated and uncoupled respiration.

3.1.5 Mitochondrial ROS production

Fibers (about 0.5mg dry) bathed in specify volume solution Z (in mM: 110 K-MES, 35 KCl, 1 EGTA, 5 K_2HPO4, 3 $MgCl_26H_2O$, 0.5mg/ml BSA, 1.2U/ml horseradish peroxidase, pH 7.3, 37°C) supplemented with the fluorescent probe Amplex Red(20µM: excitation-emission: 563-587nm) were used to determine mitochondrial hydrogen peroxide (H_2O_2) release(Ascah, Khairallah et al. 2011). Release of H_2O_2, was measured by fluorescence spectroscopy in quartz microcuvettes under continuous magnetic steering. Fluorescence was first read at baseline and substrates were then added sequentially as follows: glutamate (5mM); succinate (5mM); ADP (10mM); and antimycin-A (8µM).

3.1.6 Calcium rentention capacity (CRC)

Ghost fibers (about 0.5mg dry) incubated at 23°C bathed in specify volume CRC solution (in mM: 250 sucrose, 10 MOPS, 0.005 EGTA, 10 Pi-Tris, pH 7.3) in a quartz microcuvette with continuous magnetic stirring. To this was added: glutamate-malate (GM) (5:2.5mM) and 0.5nM oligomycin and the fluorescent probe calcium green (5N: excitation –emission: 505-535nm) in order to determine calcium retention capacity of mitochondria(Picard, Csukly et al. 2008). After adding the previous substrates, a pulse of 20 nmol calcium (Ca^{2+}) was added to determine CRC per mg of dry fiber weight in the cuvette,. CRC refers to the amount of calcium accumulated in mitochondria before their PTP opens and releases it. Concentration

of calcium was calculated in relation to the standard curve relating calcium concentration to fluorescence of calcium-green.

3.1.7 Enzymology

The enzymatic activities of citrate synthase (CS), aconitase, complex I, complex II, complex I+III, and complex IV were measured by spectrophotometry on a plate reader using enzyme-coupled assays (Marcil, Ascah et al. 2006, de Wit, Scholte et al. 2008). The enzymatic activities were all given in mU/min/mg of protein except for aconitase, which was expressed in mU/min/mg/mg tissue. Each complex is given a specific substrate enabling us to read absorption differences over a period of time.

Citrate synthase (CS) consists of the first enzyme in the Krebs cycle that transforms acetyl-CoA and oxaloacetate into citrate and releases CoA-SH. The change in absorbance of DTNB at 412nm depicts the CS activity and the buffer used consisted of 100mM Tris 200mM, 200mM Acetyl CoA, 200mM DTNB, 1% Triton X-100 10% and water. The disulfure brigde of DTNB is cleaved by the thiol part of CoA-SH and produce 2 NTBs. These DTTBs ionise in water and emit a yellow coloration that can absorb light at 412nm. The molecular coefficient of extinction of DTNB is 13.6 Lmol-1cm-1.

Equation: $Activity\ CS\ in\ mU.mg^{-1} = \left[\frac{(Slope*\Delta D)}{(\varepsilon M \times l)}\right] \times \left[\frac{V}{m}\right]$

Complex 1 (CI) activity is measured by changes in absorbance of NADH at 340nm. Complex 1 oxydizes NADH into NAD^+. The buffer used consisted of 50mM-

2mM of KPi buffer 50mM, 1mg/mL BSA, 250mM KCN, 200mM NADH, 0.4mM Antimycine A (AA) and 100mM of Decylubiquinone. In order to isolate CI, AA and KCN inhibit CIII and CIV respectively. Finally, CI is inhibited with 20mM rotenone at the end of the reaction to correct for CI-independent NADH oxidation. CI enzymatic activity is the difference between the two slopes obtained. The equation is the same as the one used to calculate CS and the molar coefficient of extinction of NADH at 340nm is 6220 $Lmol^{-1}cm^{-1}$.

The activity of Complex 2 (CII) corresponds to the variation of absorption of DCIP @ 600nm. The buffer used to measure the enzymatic activity of CII consists of 50mM-2mM of KPi-EDTA tampon 50mM-2mM, 1mg/mL BSA, 240mM, Rotenone 4mM, AA 0.4mM, 100mM Decylubiquinone, DCIP 100mM, Succinate 10mM and ATP 2mM. The oxidation of succinate and decylubiquinone by CII enables the reduction of DCIP. The electron flux is inhibited by inhibition of CI by rotenone and inhibition of CIV by KCN. Malonate 10mM is then added to inhibit CII and correct the slopes by measuring the autoreduction of DCIP. The molar coefficient of extinction of DCIP is 16.3 $Lmol^{-1}$ cm^{-1} and the equation to calculate the enzymatic activity of CII is the same as the one for CS.

The activity of complex I+III consists of the increase of absorbance of cytochrome c due to its reduction. The buffer used consists of KPI, BSA 1mg/ml, NADH 0.8mM, cytochrome c 40uM, 0.4mM KCN and rotenone 4uM. NADH donates

electrons to complex I before being transferred to complex III, which enables the latter to reduce cytochrome c. The KCN is used to inhibit complex IV and prevent the oxidation of cytochrome c, which has a molar coefficient of extinction of $29.5 mM^{-1}$. Rotenone is also added in order to block complex I. The difference in the slopes prior and after adding rotenone represents the enzymatic activity of complex I+III.

CIV activity is measured by the decrease in absorbance of oxidized cytochrome c by CIV. To get this measurement, we must start by giving CIV reduced and purified cytochrome c. The buffer used to measure CIV is composed of 50mM KPi buffer 25mM, dodecylmaltoside 0.1% and cytochrome c 100mM. Finally, the molar coefficient of extinction of DCIP is 29.5 $lmol^{-1}cm^{-1}$ and the equation is once again the same as the one to calculate CS.

3.1.8 Statistical analysis

All results are expressed as mean ± SEM. For statistical analysis 2-way ANOVA tests were used to compare the effect of the genotype and doxorubicin treatment. Bonferonni's multiple comparison tests were performed to determine where the differences were located. A P value of $P<0.05$ was considered statistically different. All analyses were performed using Prism 6.0 (GraphPad Sofware).

3.2 Results

3.2.1 Echocardiography and morphometric analyses

Figure 6 summarizes the impact of Parkin deficiency and doxorubicin administration on mice morphometric parameters. At baseline, Parkin-deficient mice had a 9.5% smaller body weight compared to the WT mice (p=0.049*). The weight of the heart, liver and lungs also tended to be lower in Parkin-deficient mice, consistent with the lower body weight. In WT mice, treatment with doxorubicin led to an almost significant 1.2 % loss of body weight (p=0.06). Heart, lung and liver weights were not significantly changed following treatment with doxorubicin. As opposed to WT mice, treatment with doxorubicin in parkin-deficient mice did not show a reducing trend in variation of body weight. After treatment with doxorubicin in WT mice, only body weight showed a strong reducing trend, while doxorubicin had no noteworthy effect in Parkin-deficient mice.

Figure 7 presents results from preliminary echocardiography analyses. At baseline, we observed no difference between WT mice and Parkin-deficient mice in terms of their cardiac index and ejection fraction, which is in agreement with our previous results obtained with Millar catheterization (Piquereau, Godin et al. 2013). End systolic and diastolic volumes tended to be lower in Parkin-deficient mice, but no significant difference could be detected with the limited sample size available (n=3 in each group). After treatment with doxorubicin, there were no significant differences in the cardiac index and ejection fraction in both WT and Parkin-deficient mice. However, WT mice displayed a reducing trend of left end-systolic and diastolic

ventricular volume following treatment with doxorubicin, while these changes were less apparent in Parkin-deficient mice, likely due to the variability observed in baseline volumes. Mitochondrionopathy can precede cardiotoxicity in certain cases preventing the observation of cardiac dysfunction following treatment with doxorubicin (Pereira, Pereira et al. 2012). This lack of effect was also demonstrated in the lab after raising the n number.

Figure 6: Effect of doxorubicin on morphometric parameters in WT and Parkin -/- mice

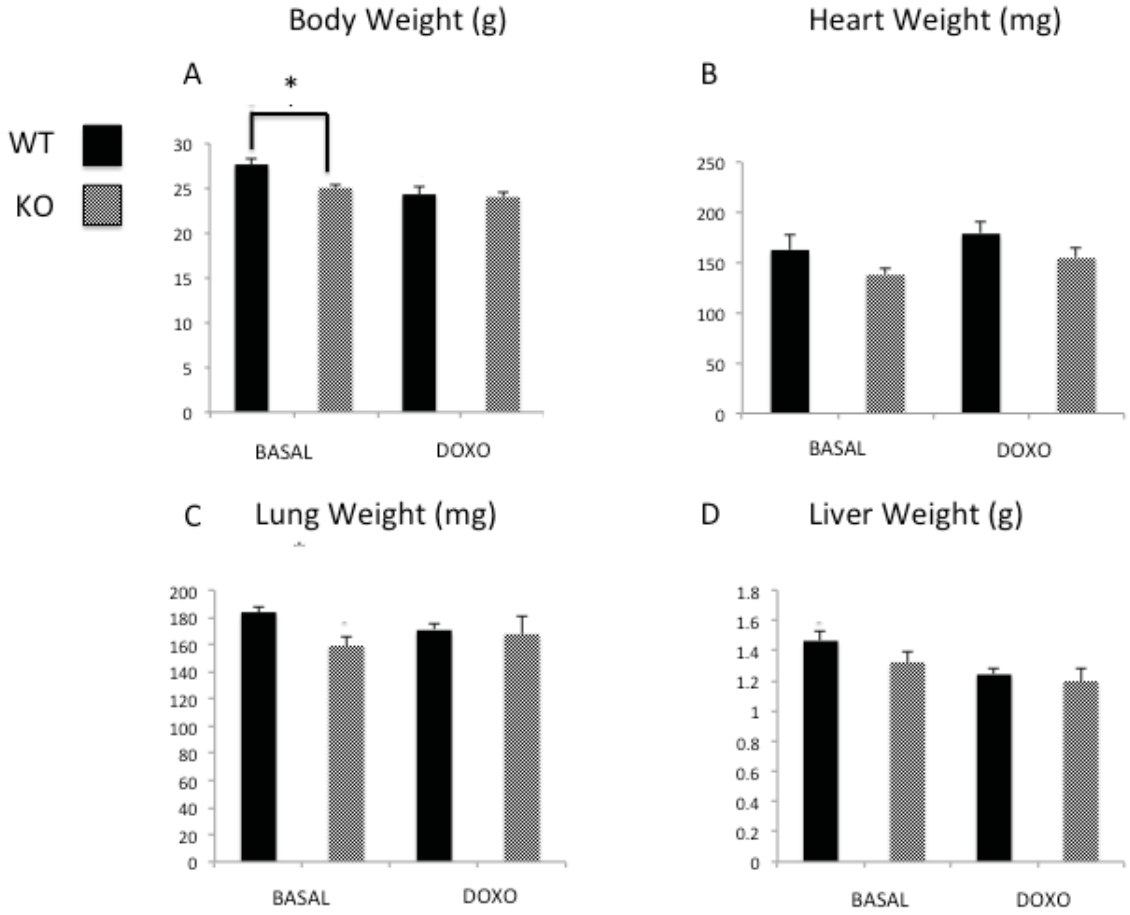

Figure 6: Effect of doxorubicin on morphometric parameters in WT and Parkin -/- mice. Doxorubicin's effect on body weight (A) (n=8-11), heart weight (B) (n=5-8), lung weight (C) (n=3-6) and liver weight (D) (n=3-6) were measured in both WT and parkin -/- mice. 15mg/kg of doxorubicin was administered by intraperitoneal injection. *, significantly different within same experimental conditions ($P<0.05$).

Figure 7: Effect of doxorubicin on cardiac function parameters in WT and Parkin-/- mice

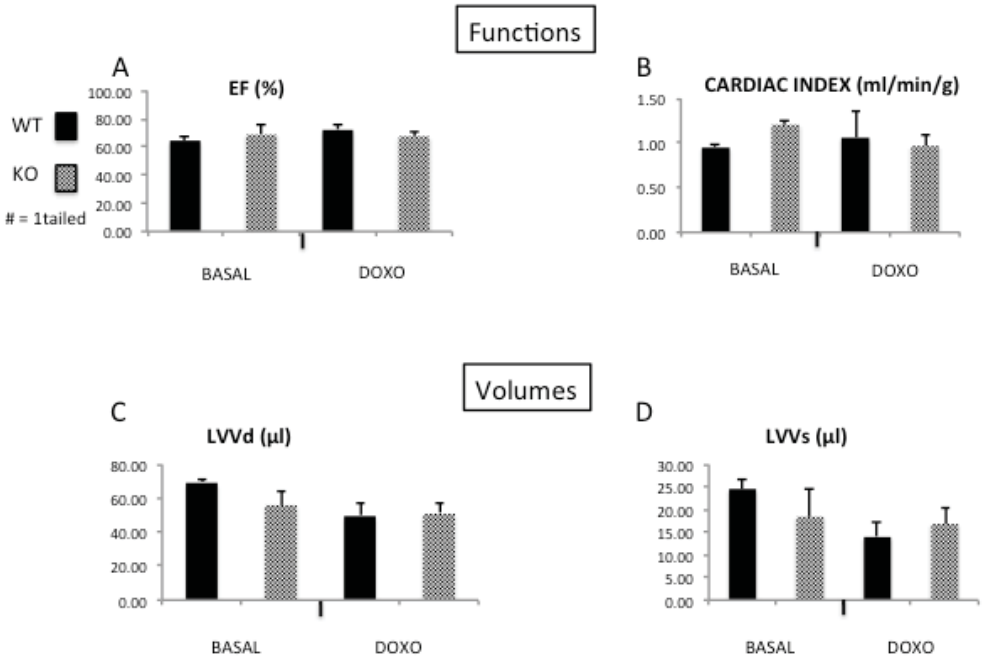

Figure 7: Effect of doxorubicin on cardiac function parameters in WT and Parkin-/- mice. Doxorubicin's ejection fraction (A), cardiac index (B), left ventricle volume in diastole (C) and systole in both WT and parkin -/- mice (n=3).

3.2.2 Mitochondrial Respiration

Following these initial observations, experiments were conducted to assess the impact of Parkin ablation and doxorubicin treatment of various aspects of mitochondrial function. We first measured mitochondrial respiration in cardiac permeabilized fibers to study different respiratory states in presence of substrates feeding specific complexes of the electron transport chain. As shown in Figure 8, at baseline levels, there was a significant reduction in most respiratory states in Parkin-deficient mice. In Parkin-deficient mice, respiration with substrates feeding complex

I stimulated by ADP (p=0.0077**), complex II (0.0064*), and complex IV (p=0.0090**) were all significantly lower than in their WT counterparts. Respiration at the level of complex I stimulated by glutamate-malate (GM) (p=0.052*) was nearly significantly smaller as well. In mice treated with doxorubicin, we observed a systematic reduction in respiration in mitochondria from WT mice (CI:p=0.0216*; ADP stimulated CI: p=0.0067** or CII: p=0.0029**; CIV: p=0.0066**). However, in Parkin-deficient mice, treatment with doxorubicin had no negative impact on respiration under all conditions. The effects of doxorubicin were completely abolished in Parkin -/- mice.

Figure 8: Effect of doxorubicin on mitochondrial respiration in WT and Parkin-/- mice

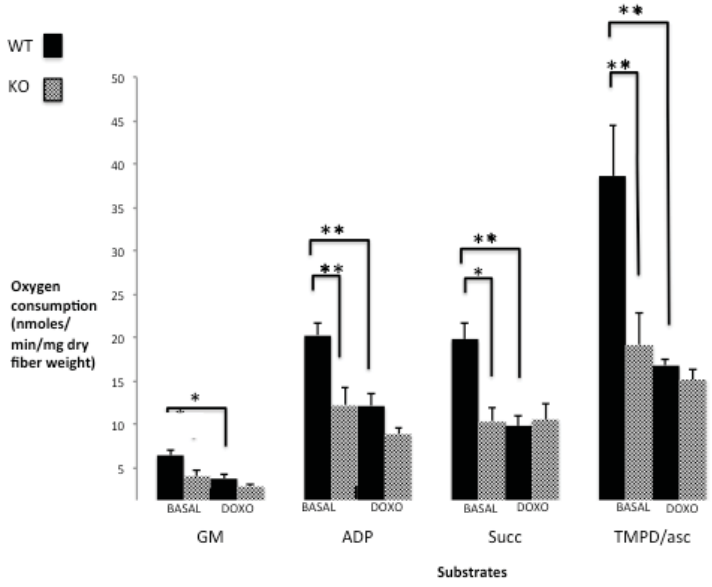

Figure 8: Effect of doxorubicin on mitochondrial respiration in WT and Parkin-/- mice. Rates of respiration of WT and KO mice treated and not treated with doxorubicin following sequential addition of: complex I substrates glutamate-malate (GM)(5, 2.5mM) (n=6-7); ADP (2mM) (n= 6); complex I inhibitor amytal (2mM) (not shown); the complex II substrate succinate (5mM) (n=4-6); chain uncoupler CCCP (1μM) (not shown); complex III inhibitor antimycin-A (AA) (8μM) (not shown); and the complex IV substrates N, N', N'- tetramethyl-p-phenylenediamine dihydrochloride (TMPD)-ascorbate (0.9mM, 9mM)(n=3-6); complex IV KCN (0.6mM) (not shown). *, significantly different within same experimental conditions ($P<0.05$). **, significantly different ($P<0.01$).

3.2.3 Enzymology

We then measured enzymatic activity of citrate synthase, aconitase, and complexes I, II, I+III, and IV of the respiratory chain. At baseline, enzymatic activity of complexes I, and I+III showed a reducing trend in Parkin-deficient mice in comparison to WT mice. However, the activity of citrate synthase, complexes II and IV, and aconitase, was not different between the two groups (Figure 9 and 10). In WT mice, treatment with doxorubicin induced a reducing trend in the enzymatic activity of complexes I and I+III, while the activities of CS, complex II, and complex IV were not affected. As expected, doxorubicin treatment also induced a reduction in aconitase activity in the heart of WT mice ($p=0.0270^*$). In contrast, in Parkin-deficient mice, treatment with doxorubicin did not significantly worsen the activity of these enzymes beyond the levels observed.

Figure 9: Effect of doxorubicin on citrate synthase and enzyme activity of complexes of the ETC in WT and Parkin-/- mice

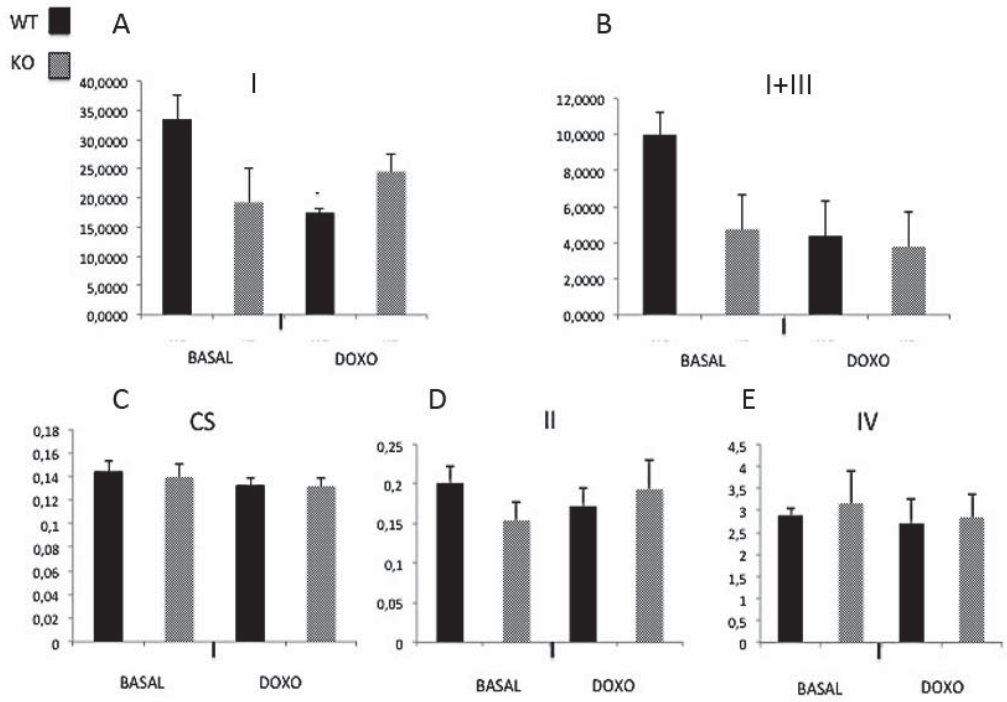

Figure 9: Effect of doxorubicin on citrate synthase and enzyme activity of complexes of the ETC in WT and Parkin-/- mice. Enzymatic activities in mU/min/mg tissu of different complexes I (A) (n=4), I+III (B) (n=4-5), CS (C) (n=4-6), II (D) (n=4-6) and IV (E) (n=4-6) of the ETC for both WT and KO mice in basal conditions and treated with doxorubicin. *, significantly different within same experimental conditions ($P<0.05$).

Figure 10: Effect of doxorubicin on aconitase activity in WT and Parkin-/- mice

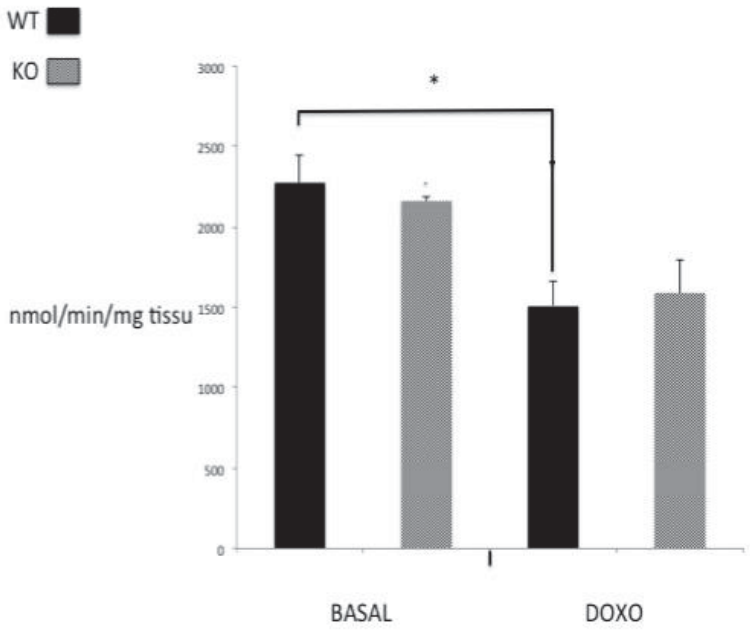

Figure 10: Effect of doxorubicin on aconitase activity in WT and Parkin-/- mice. Aconitase activity in WT and KO mice (n=4) in basal and doxorubicin treated conditions. *, significantly different within same experimental conditions ($P<0.05$).

3.2.4 Mitochondrial H_2O_2 release

Mitochondrial H_2O_2 release per unit of oxygen consumed was directly measured using the Amplex red probe. While significant differences were observed under different conditions tested, we focus solely on when the respiratory chain was fed with the complex II substrate succinate, which maximizes ROS production by promoting reverse electron flow through complex I. As shown in Figure 11, under baseline conditions, mitochondrial H_2O_2 release was systematically greater in Parkin-

deficient mice compared to WT mice under all conditions tested but significant differences were evident when the respiratory chain was fed with the complex II substrate succinate ($p=0.0108$). In WT mice, treatment with doxorubicin induced a significant increase in H_2O_2 release ($p<0.0001$), particularly in presence of the complex II substrate succinate. In contrast, in Parkin-deficient mice, the increase in H_2O_2 release following doxorubicin treatment was blunted in presence of succinate.

Figure 11: Effect of doxorubicin on mitochondrial H_2O_2 release in WT and Parkin-/- mice

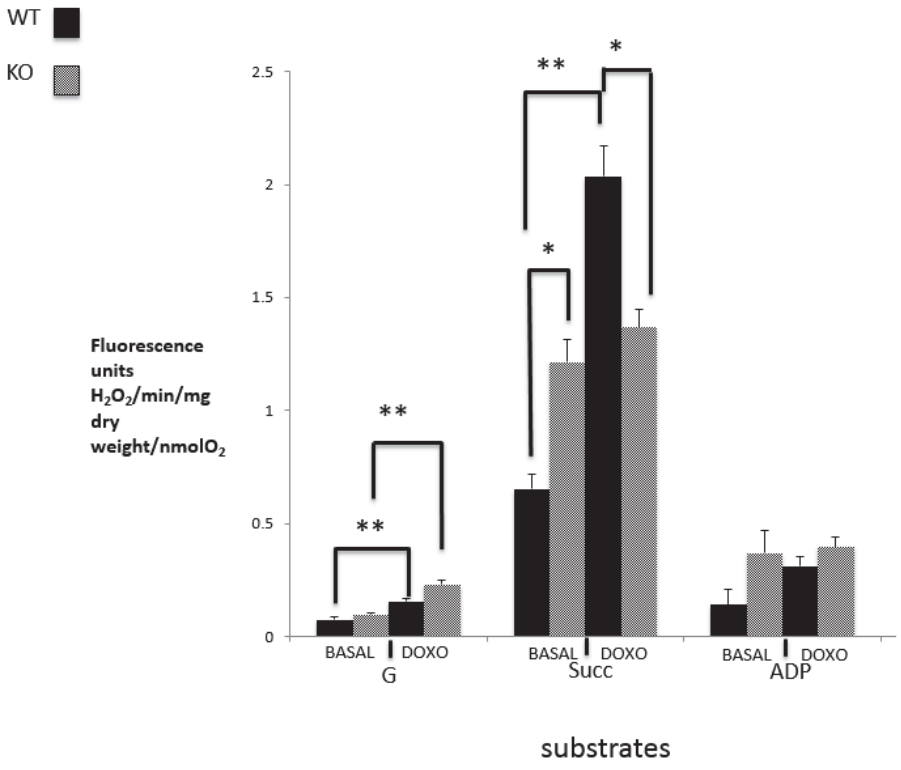

Figure 11: Effect of doxorubicin on mitochondrial H_2O_2 release in WT and Parkin-/- mice. Hydrogen peroxide production per unit of oxygen consumed in WT and KO mice in basal conditions and post-treatment with doxorubicin following sequential addition of glutamate (5mM) (n=7-11); succinate (5mM) (n=6-11); ADP (10mM) (n=7-11); and antimycin-A (8μM) (not shown). The data was normalized by oxygen consumed in order to accurately depict the correlation between oxygen consumed and ROS produced. *, significantly different within same experimental conditions ($P<0.05$). **, significantly different ($P<0.01$).

3.2.5 Calcium Retention Capacity

We also examined the susceptibility of mitochondria to Ca^{2+}-induced opening of the permeability transition pore (PTP). At baseline, susceptibility to PTP opening was greater in Parkin-deficient mice in comparison to WT mice, as evidenced by lower calcium retention capacities (p=0.0477*) and time to pore opening was nearly

significantly lower than it's basal WT counterpart (p=0.0504). In WT mice, treatment with doxorubicin significantly enhanced sensitivity to PTP opening (CRC: p=0.0259*, time to opening: p=0.098**). However, in Parkin-deficient mice, susceptibility to PTP opening was not deteriorated beyond levels observed at baseline (12).

Figure 12: Effect of doxorubicin on PTP opening in WT and Parkin-/- mice

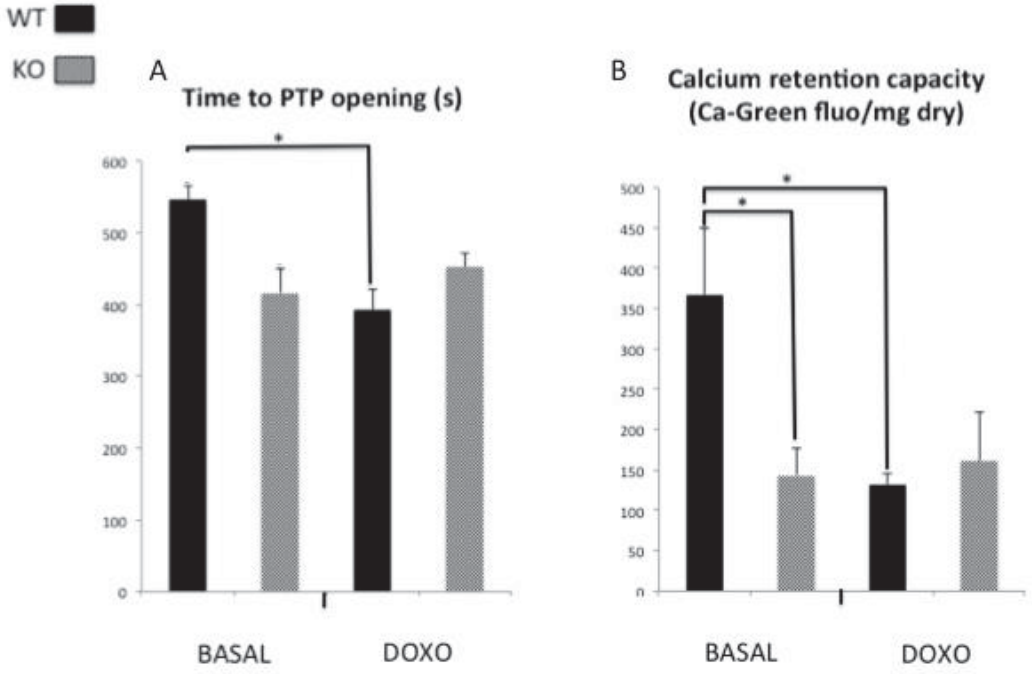

Figure 12: Effect of doxorubicin on PTP opening in WT and Parkin-/- mice
Time necessary for opening of PTP (n=4-7) and calcium retention capacity (CRC) (n= 5-6) of WT and KO mice in both basal situation and following treatment with doxorubicin. *, significantly different within same experimental conditions ($P<0.05$).

3.3 Discussion

Mitochondrial quality control mechanisms are increasingly considered as being central determinant of mitochondrial health in mammalian tissues, particularly in energy demanding tissues such as the heart where these organelles are abundant. In the present study we aimed to determine whether selective autophagy of mitochondria (*i.e.* mitophagy) through the Parkin-dependent pathway is required for the maintenance of normal mitochondrial and cardiac function, and important to mount adequate response to mitochondrial stress in a well-characterized model of antracyclin-induced cardiotoxicity. Although mitochondrial quality control was not formally investigated, it currently represents the main factor linking Parkin to mitochondrial function. Our results show that genetic inactivation of Parkin at the germline level does not induce overt cardiopathy under normal baseline conditions, but nevertheless causes multiple but moderate mitochondrial dysfunctions, suggesting that Parkin participates in baseline mitochondrial quality control. As expected, *in vivo* treatment with doxorubicin induced multiple mitochondrial abnormalities in wild type mice that were clearly observable in absence of heart failure. However, in contrast to our initial hypothesis, genetic inactivation of Parkin did not enhance mitochondrial dysfunctions induced by doxorubicin, and even seemed to exert a protective effect, through mechanisms that remain to be elucidated.

3.3.1 Role of Parkin in the maintenance of baseline cardiac and mitochondrial phenotype

Under baseline conditions, we observed that the absence of Parkin did not significantly impair cardiac function. Both the cardiac index and the ejection fraction showed no difference in Parkin-deficient mice in comparison to WT mice. In terms of cardiac volumes, both the end systolic and end diastolic volumes tended to be lower in Parkin deficient mice compared to controls, but the difference did not reach statistical significance, possibly due to the small sample size inherent to these preliminary experiments. However, despite the absence of a clear cardiac phenotype, the absence of Parkin was nevertheless associated with multiple mitochondrial dysfunctions such as impairment of mitochondrial respiration, increased production of reactive oxygen species and reduced calcium retention capacity, which is indicative of greater susceptibility to PTP opening. These baseline results are in line with previous findings from our laboratory (Piquereau, Godin et al. 2013). It is interesting to note however, that a previous study by another laboratory reported no difference in baseline mitochondrial functions in Parkin-deficient mice compared to controls (Kubli, Zhang et al. 2013). Although the reasons underlying this difference are unclear, one possibility is that in this latter study, measurements were performed on isolated mitochondria. The use of this more disruptive preparation could explain the differences with our laboratory's results.

3.3.2 Impact of Doxorubicin on cardiac and mitochondrial function

As expected, treatment with doxorubicin resulted in mitochondrial dysfunctions at multiple levels. Our study and previously published experiments (Yen, Oberley et al. 1999) (Santos, Moreno et al. 2002) (Serrano, Palmeira et al. 1999) confirmed the ability of doxorubicin to inhibit mitochondrial respiration and trigger a significant rise in ROS production leading to oxidative stress and reduction in the threshold for calcium induced PTP opening. Interestingly, most previous studies (Zhang, El-Sikhry et al. 2009) have reported these mitochondrial abnormalities at significantly advanced stages of cardiopathy at a time when hemodynamic function was impaired. In contrast, in our study, our preliminary echocardiography results suggest that we were able to observe these changes prior to overt signs of cardiac dysfunction. This suggests that mitochondrial dysfunctions are not simply a consequence of heart failure but rather, a pathogenic mechanism.

3.3.3 Impact of Parkin deficiency in response to doxorubicin.

In response to treatment with doxorubicin, our study indicates that Parkin deficiency did not worsen mitochondrial functions and if anything, suggested a protective effect. In terms of morphometric analysis, Parkin deficiency blunted the effect of doxorubicin on animal weight loss. Decline in cardiac mitochondrial functions and enzymology were also not observed in Parkin deficient mice treated with doxorubicin although comparison to WT mice is partly confounded by the fact that Parkin-KO hearts had lower mitochondrial functions to begin with. There is also the possibility of a ceiling effect of the KO that may mask the effect of doxorubicin.

While we do not have a definite explanation for this, as discussed below, we believe that these data cannot be simply interpreted as an indication that mitophagy is detrimental in the context of doxorubicin-induced cardiotoxicity.

3.3.3.1 Germline knockout of the Park2 gene produces a complex phenotype:

Compensatory mechanisms add an important degree of complexity to the study of specific quality control mechanisms such as mitophagy. Studies in our laboratory as well as other previously published experiments (Hsieh, Pai et al. 2011) have reported activation of macro-autophagy in myocytes following sepsis induction. There are two types of compensatory mechanisms related to mitophagy: i) activation of alternate mechanisms of mitophagy and ii) enhanced macro-autophagy.

To support the role of alternate mitophagy mechanism, our laboratory has previously demonstrated that there is more LC3-II recruited to mitochondria in Parkin-deficient hearts compared to WT (Piquereau, Godin et al. 2013). This not only suggests the existence of compensatory mechanisms that can protect Parkin-deficient mice, but it also suggests the existence of other mitophagic mechanisms then the Parkin/Pink-1 pathway. As suggested by others, alternate E-3 ligases may compensate for Parkin-deficiency (Ding, Ni et al. 2010). In yeast, mitophagy can also be achieved via a ubiquitin-independent pathway with direct binding of ATG8 family of mitochondrial autophagy receptors. In yeast, this pathway requires ATG32 receptor (Kanki, Wang et al. 2009) while in mammalian cells, NIX and BNIP3 are mitochondrial autophagy receptors (Novak, Kirkin et al. 2010). It is notable that both

NIX and BNIP3 are recruited to mitochondria in WT and Parkin-KO hearts in response to sepsis (Piquereau, Godin et al. 2013). Altogether, these results thus suggest the possibility that Parkin ablation in the heart does not overtly compromise stress-induced mitophagy. However, this does not really contribute to explain how Parkin deficiency seems to attenuate the negative impact of doxorubicin observed in the present study.

In terms of enhanced macro-autophagy, our laboratory was recently first to report that Parkin-deficiency is associated with an upregulation of macro-autophagy (Piquereau, Godin et al. 2013). In fact, baseline transcripts of major autophagy-regulated genes were found to have increased expression in Parkin-deficient mice when compared to WT. Moreover, autophagy flux measured *in vivo* by the accumulation of LC3-II, was significantly higher in Parkin-KO hearts both at baseline and in response to LPS treatment (Piquereau, Godin et al. 2013). Morphological experiments also confirmed the increased number of autophagosomes in Parkin-deficient mice hearts in comparison to WT mice. Also, large autophagosomes containing mitochondrial remnants, damaged cellular material and lipid droplets were found in Parkin KO mice but not in WT mice following LPS treatment (Piquereau, Godin et al. 2013). Together these data suggested that genetic inactivation of Parkin at the germline level results in a compensatory upregulation of less specific forms of autophagy. As discussed below, we believe that this constitutive

activation of macro-autophagy may contribute to explain the paradoxically beneficial effect of Parkin-deficiency in response to doxorubicin.

Several studies were recently performed to examine the role of macro-autophagy in the heart in response to stress. In mouse models, evidence suggest that doxorubicin blunts the autophagic process, which in turns contribute to the cardiopathic phenotype. More specifically, Kawaguchi et al. reported that doxorubicin treatment leads to the accumulation of several autophagy makers in adult mouse hearts (LC3-II, P62 and Cathepsin D) and neonatal cardiomyocytes (Kawaguchi, Takemura et al. 2012). However, several evidence indicate that this accumulation is caused by reduced autophagosome clearance rather than activation of autophagosome formation: *i)* blockade of the lysosomal degradation by cloroquine did not further enhance LC3-II accumulation, ii) doxorubicin administration in fact inhibited AMPK-ULK1, a major trigger of autophagy. Moreover, starvation prior to doxorubicin administration was able to mitigate Doxorubicin-induced cardiac dysfunction, which was associated with a lower accumulation of LC3-II puncta and restoration of AMPK-ULK1 activation, suggesting that re-activation of autophagy was beneficial. Studies performed *in vitro* in H9C2 cardioblasts also reported that the autophagy inducer rapamycin mitigated doxorubicin-induced cellular toxicity (Kawaguchi, Takemura et al. 2012) (Sishi, Loos et al. 2013). Therefore, it is tempting to speculate that in our study, constitutive activation of macro-autophagy contributes to explain the apparently protective effect of Parkin-deficiency in mice treated with

doxorubicin. However, this hypothesis remains to be tested by measuring the autophagy flux of the different groups.

It should be mentioned that conflicting results exist with respect to the effect of doxorubicin treatment on cardiac autophagy. Indeed, several studies performed in rat models suggest that doxorubicin treatment results in excessive activation of autophagy, which in turn promotes cardiac dysfunction (Lu, Wu et al. 2009) Chen, Kobayashi et al. 2009). Indeed, a study by the group of Lu et al. demonstrated that doxorubicin stimulated cardiac autophagy (Lu, Wu et al. 2009). Rat neonatal cardiomyocytes treated with doxorubicin showed an increase in the number of autophagic vesicles. Another group also reported that autophagy was upregulated following treatment with doxorubicin (Dimitrakis, Romay-Ogando et al. 2012). Similar to Lu et al., the latter group concluded doxorubicin resulted in excessive activation of autophagy based on the enhanced accumulation of autophagosomes following blockage of lysosomal degradation with cloroquine. Two other studies also reported evidences suggesting excessive activation of autophagy in rat models of doxorubicin cardiotoxicity.

Kobayashi et al (Kobayashi, Volden et al. 2010) demonstrated a clear increase in autophagic flux (LC3-II/I) in cells treated with doxorubicin. They also found an increase in the number of autophagic vesicles and Beclin1 resulting in an increase in the cardiotoxicity induced by doxorubicin. Kobayashi also analyzed cell death when

doxorubicin was administered with rapamycin (stimulator of autophagy) and 3MA (autophagy inhibitor). Rapamycin in conjunction with doxorubicin caused an increase in cell death while 3MA was protective. Finally, another study (Smuder, Kavazis et al. 2013)performed in rat hearts reported that doxorubicin administration stimulated the LC3 flux and enhanced the expression of several autophagy related genes (Atg12, Atg12-5, Atg7, Atg4).

Currently, the only hypothesis to explain the conflicting data with respect to the effect of doxorubicin on cardiac autophagy is inter-species differences, i.e. doxorubicin blocks and over stimulates autophagy in mice and rats respectively (Dirks-Naylor, 2013). Overall, results obtained in Parkin-deficient mice following treatment with doxorubicin have thus revealed a phenotype that is more complex than initially expected. Future studies are needed to provide more definite explanations. Importantly, strategies such as the use of cardiac-specific inducible Parkin knockout models should be used to try to limit the occurrence of developmental compensation such as upregulation of macro-autophagy (Dimitrakis, Romay-Ogando et al. 2012). This would allow to more specifically test the role of mitophagy in the cardiac response to doxorubicin. In addition, a more direct investigation of mitophagy and macro-autophagy should be performed to directly confirm the impact of doxorubicin on these quality control mechani

4 Conclusions

In mammalian tissues, mitochondrial quality control mechanisms play an important role in mitochondrial health. The objective of our study was to determine if Parkin-dependent mitophagy was necessary in the maintenance of normal mitochondria and cardiac function in response to doxorubicin-induced cardiotoxicity. In line with previous findings in our laboratory, at baseline, the absence of Parkin did not induce a cardiac phenotype, but was associated with several mitochondrial dysfunctions such as respiratory impairment, elevated production of ROS and greater susceptibility to calcium-induced PTP opening; advocating for the fact that at baseline, Parkin contributes the maintenance of normal mitochondrial function. Similarly, doxorubicin also induced multiple mitochondrial dysfunctions in wild type mice that preceded cardiac dysfunctions. This suggests that mitochondrial dysfunctions might represent a pathological mechanism as opposed to a consequence of doxorubicin-induced cardiotoxicity. Finally, in contrast to our hypothesis, the absence of Parkin did not accentuate mitochondrial dysfunctions following treatment with doxorubicin and even seemed to have a certain protective effect. Several factors could contribute to explain this peculiar observation. First, it is possible that since our parkin-deficient mice are already negatively impacted at baseline level, the subsequent consequences of treatment with doxorubicin cannot be appropriately measured. Second, the existence of compensatory mechanisms, particularly the constitutive activation of macro-autophagy in Parkin knockout mice could afford a

certain level of protection considering that in mice models, impaired autophagy is suggested to contribute to doxorubicin-induced cardiomyopathy. However, considering the existing debate on the role the response of cardiac autophagy to anthracyclins, further work is required to elucidate this question

5 BIBLIOGRAPHY

Arafa, M. H., N. S. Mohammad, H. H. Atteia and H. R. Abd-Elaziz (2014). "Protective effect of resveratrol against doxorubicin-induced cardiac toxicity and fibrosis in male experimental rats." J Physiol Biochem **70**(3): 701-711.

Ascah, A., M. Khairallah, F. Daussin, C. Bourcier-Lucas, R. Godin, B. G. Allen, B. J. Petrof, C. Des Rosiers and Y. Burelle (2011). "Stress-induced opening of the permeability transition pore in the dystrophin-deficient heart is attenuated by acute treatment with sildenafil." Am J Physiol Heart Circ Physiol **300**(1): H144-153.

Aversano, R. C. and P. J. Boor (1983). "Histochemical alterations of acute and chronic doxorubicin cardiotoxicity." J Mol Cell Cardiol **15**(8): 543-553.

Axe, E. L., S. A. Walker, M. Manifava, P. Chandra, H. L. Roderick, A. Habermann, G. Griffiths and N. T. Ktistakis (2008). "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum." J Cell Biol **182**(4): 685-701.

Baines, C. P., R. A. Kaiser, N. H. Purcell, N. S. Blair, H. Osinska, M. A. Hambleton, E. W. Brunskill, M. R. Sayen, R. A. Gottlieb, G. W. Dorn, J. Robbins and J. D. Molkentin (2005). "Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death." Nature **434**(7033): 658-662.

Ballinger, S. W. (2005). "Mitochondrial dysfunction in cardiovascular disease." Free Radic Biol Med **38**(10): 1278-1295.

Bernardi, P. (2013). "The mitochondrial permeability transition pore: a mystery solved?" Front Physiol **4**: 95.

Bernardi, P., A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa and M. A. Forte (2006). "The mitochondrial permeability transition from in vitro artifact to disease target." FEBS J **273**(10): 2077-2099.

Beutner, G., A. Ruck, B. Riede and D. Brdiczka (1998). "Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases." Biochim Biophys Acta **1368**(1): 7-18.

Bonnefoy-Berard, N., A. Aouacheria, C. Verschelde, L. Quemeneur, A. Marcais and J. Marvel (2004). "Control of proliferation by Bcl-2 family members." Biochim Biophys Acta **1644**(2-3): 159-168.

Brand, M. D. (2010). "The sites and topology of mitochondrial superoxide production." Exp Gerontol **45**(7-8): 466-472.

Brenner, C. and M. Moulin (2012). "Physiological roles of the permeability transition pore." Circ Res **111**(9): 1237-1247.

Cadenas, E. and K. J. Davies (2000). "Mitochondrial free radical generation, oxidative stress, and aging." Free Radic Biol Med **29**(3-4): 222-230.

Chacon, E. and D. Acosta (1991). "Mitochondrial regulation of superoxide by Ca2+: an alternate mechanism for the cardiotoxicity of doxorubicin." Toxicol Appl Pharmacol **107**(1): 117-128.

Chan, N. C., A. M. Salazar, A. H. Pham, M. J. Sweredoski, N. J. Kolawa, R. L. Graham, S. Hess and D. C. Chan (2011). "Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy." Hum Mol Genet **20**(9): 1726-1737.

Chang, N. C., M. Nguyen, M. Germain and G. C. Shore (2010). "Antagonism of Beclin 1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1." EMBO J **29**(3): 606-618.

Chen, D., W. Fan, Y. Lu, X. Ding, S. Chen and Q. Zhong (2012). "A mammalian autophagosome maturation mechanism mediated by TECPR1 and the Atg12-Atg5 conjugate." Mol Cell **45**(5): 629-641.

Chen, D., F. Gao, B. Li, H. Wang, Y. Xu, C. Zhu and G. Wang (2010). "Parkin mono-ubiquitinates Bcl-2 and regulates autophagy." J Biol Chem **285**(49): 38214-38223.

Chen, G., R. Ray, D. Dubik, L. Shi, J. Cizeau, R. C. Bleackley, S. Saxena, R. D. Gietz and A. H. Greenberg (1997). "The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis." J Exp Med **186**(12): 1975-1983.

Chen, K., X. Xu, S. Kobayashi, D. Timm, T. Jepperson and Q. Liang (2011). "Caloric restriction mimetic 2-deoxyglucose antagonizes doxorubicin-induced cardiomyocyte death by multiple mechanisms." J Biol Chem **286**(25): 21993-22006.

Chiang, H. L., S. R. Terlecky, C. P. Plant and J. F. Dice (1989). "A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins." Science **246**(4928): 382-385

Choo, Y. S., G. V. Johnson, M. MacDonald, P. J. Detloff and M. Lesort (2004). "Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release." Hum Mol Genet **13**(14): 1407-1420.

Chu, C. T., J. Ji, R. K. Dagda, J. F. Jiang, Y. Y. Tyurina, A. A. Kapralov, V. A. Tyurin, N. Yanamala, I. H. Shrivastava, D. Mohammadyani, K. Z. Qiang Wang, J. Zhu, J. Klein-Seetharaman, K. Balasubramanian, A. A. Amoscato, G. Borisenko, Z. Huang, A. M. Gusdon, A. Cheikhi, E. K. Steer, R. Wang, C. Baty, S. Watkins, I. Bahar, H. Bayir and V. E. Kagan (2013). "Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells." Nat Cell Biol **15**(10): 1197-1205.

Clark, I. E., M. W. Dodson, C. Jiang, J. H. Cao, J. R. Huh, J. H. Seol, S. J. Yoo, B. A. Hay and M. Guo (2006). "Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin." Nature **441**(7097): 1162-1166.

Cuervo, A. M., E. Bergamini, U. T. Brunk, W. Droge, M. Ffrench and A. Terman (2005). "Autophagy and aging: the importance of maintaining "clean" cells." Autophagy **1**(3): 131-140.

Davies, K. J. and J. H. Doroshow (1986). "Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase." J Biol Chem **261**(7): 3060-3067.

De Duve, C. and R. Wattiaux (1966). "Functions of lysosomes." Annu Rev Physiol **28**: 435-492.

de Wit, L. E., H. R. Scholte and W. Sluiter (2008). "Correct assay of complex I activity in human skin fibroblasts by timely addition of rotenone." Clin Chem **54**(11): 1921-1922; author reply 1922-1924.

Demant, E. J. and P. K. Jensen (1983). "Destruction of phospholipids and respiratory-chain activity in pig-heart submitochondrial particles induced by an adriamycin-iron complex." Eur J Biochem **132**(3): 551-556.

Dennemarker, J., T. Lohmuller, S. Muller, S. V. Aguilar, D. J. Tobin, C. Peters and T. Reinheckel (2010). "Impaired turnover of autophagolysosomes in cathepsin L deficiency." Biol Chem **391**(8): 913-922.

Devasagayam, T. P., J. C. Tilak, K. K. Boloor, K. S. Sane, S. S. Ghaskadbi and R. D. Lele (2004). "Free radicals and antioxidants in human health: current status and future prospects." J Assoc Physicians India **52**: 794-804.

Dimitrakis, P., M. I. Romay-Ogando, F. Timolati, T. M. Suter and C. Zuppinger (2012). "Effects of doxorubicin cancer therapy on autophagy and the ubiquitin-proteasome system in long-term cultured adult rat cardiomyocytes." Cell Tissue Res **350**(2): 361-372.

Ding, W. X., H. M. Ni, M. Li, Y. Liao, X. Chen, D. B. Stolz, G. W. Dorn, 2nd and X. M. Yin (2010). "Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming." J Biol Chem **285**(36): 27879-27890.

Dirks-Naylor, A. J. (2013). "The role of autophagy in doxorubicin-induced cardiotoxicity." Life Sci.

Dohm, G. L., E. B. Tapscott and G. J. Kasperek (1987). "Protein degradation during endurance exercise and recovery." Med Sci Sports Exerc **19**(5 Suppl): S166-171.

Donati, A. (2006). "The involvement of macroautophagy in aging and anti-aging interventions." Mol Aspects Med **27**(5-6): 455-470.

Du, J., X. Wang, C. Miereles, J. L. Bailey, R. Debigare, B. Zheng, S. R. Price and W. E. Mitch (2004). "Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions." J Clin Invest **113**(1): 115-123.

Etgen, G. J., B. A. Oldham, W. T. Johnson, C. L. Broderick, C. R. Montrose, J. T. Brozinick, E. A. Misener, J. S. Bean, W. R. Bensch, D. A. Brooks, A. J. Shuker, C. J. Rito, J. R. McCarthy, R. J. Ardecky, J. S. Tyhonas, S. L. Dana, J. M. Bilakovics, J. R. Paterniti, Jr., K. M. Ogilvie, S. Liu and R. F. Kauffman (2002). "A tailored therapy for the metabolic syndrome: the dual peroxisome proliferator-activated receptor-alpha/gamma agonist LY465608 ameliorates insulin resistance and diabetic hyperglycemia while improving cardiovascular risk factors in preclinical models." Diabetes **51**(4): 1083-1087.

Exner, N., A. K. Lutz, C. Haass and K. F. Winklhofer (2012). "Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences." EMBO J **31**(14): 3038-3062.

Frey, T. G. and C. A. Mannella (2000). "The internal structure of mitochondria." Trends Biochem Sci **25**(7): 319-324.

Gegg, M. E., J. M. Cooper, K. Y. Chau, M. Rojo, A. H. Schapira and J. W. Taanman(2010). "Mitofusin 1 and mitofusin 2 are ubiquitinated in a

PINK1/parkin-dependent manner upon induction of mitophagy." Hum Mol Genet **19**(24): 4861-4870.

Geisler, S., K. M. Holmstrom, D. Skujat, F. C. Fiesel, O. C. Rothfuss, P. J. Kahle and W. Springer (2010). "PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1." Nat Cell Biol **12**(2): 119-131.

Gewirtz, D. A. (1999). "A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin." Biochem Pharmacol **57**(7): 727-741.

Gottlieb, R. A., K. D. Finley and R. M. Mentzer, Jr. (2009). "Cardioprotection requires taking out the trash." Basic Res Cardiol **104**(2): 169-180.

Greene, J. C., A. J. Whitworth, I. Kuo, L. A. Andrews, M. B. Feany and L. J. Pallanck (2003). "Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants." Proc Natl Acad Sci U S A **100**(7): 4078-4083.

Grenier, K., G. L. McLelland and E. A. Fon (2013). "Parkin- and PINK1-Dependent Mitophagy in Neurons: Will the Real Pathway Please Stand Up?" Front Neurol **4**: 100.

Gustafsson, A. B. and R. A. Gottlieb (2008). "Eat your heart out: Role of autophagy in myocardial ischemia/reperfusion." Autophagy **4**(4): 416-421.

Han, D., E. Williams and E. Cadenas (2001). "Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space." Biochem J **353**(Pt 2): 411-416.

Hanna, R. A., M. N. Quinsay, A. M. Orogo, K. Giang, S. Rikka and A. B. Gustafsson (2012). "Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy." J Biol Chem **287**(23): 19094-19104.

Harrison, R. E., C. Bucci, O. V. Vieira, T. A. Schroer and S. Grinstein (2003). "Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP." Mol Cell Biol **23**(18): 6494-6506.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori and A. Yamamoto (2009). "A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation." Nat Cell Biol **11**(12): 1433-1437.

Hsieh, C. H., P. Y. Pai, H. W. Hsueh, S. S. Yuan and Y. C. Hsieh (2011). "Complete induction of autophagy is essential for cardioprotection in sepsis." Ann Surg **253**(6): 1190-1200.

Huart, P., R. Brasseur, E. Goormaghtigh and J. M. Ruysschaert (1984). "Antimitotics induce cardiolipin cluster formation. Possible role in mitochondrial enzyme inactivation." Biochim Biophys Acta **799**(2): 199-202.

Inoue, M., E. F. Sato, M. Nishikawa, A. M. Park, Y. Kira, I. Imada and K. Utsumi (2003). "Mitochondrial generation of reactive oxygen species and its role in aerobic life." Curr Med Chem **10**(23): 2495-2505.

Ishihara, N. and N. Mizushima (2009). "A receptor for eating mitochondria." Dev Cell **17**(1): 1-2.

Itakura, E. and N. Mizushima (2010). "Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins." Autophagy **6**(6): 764-776.

Itakura, E. and N. Mizushima (2011). "p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding." J Cell Biol **192**(1): 17-27.

Jager, S., C. Bucci, I. Tanida, T. Ueno, E. Kominami, P. Saftig and E. L. Eskelinen (2004). "Role for Rab7 in maturation of late autophagic vacuoles." J Cell Sci **117**(Pt 20): 4837-4848.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi and T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J **19**(21): 5720-5728.

Kang, R., H. J. Zeh, M. T. Lotze and D. Tang (2011). "The Beclin 1 network regulates autophagy and apoptosis." Cell Death Differ **18**(4): 571-580.

Kanki, T., K. Wang, Y. Cao, M. Baba and D. J. Klionsky (2009). "Atg32 is a mitochondrial protein that confers selectivity during mitophagy." Dev Cell **17**(1): 98-109.

Kassiotis, C., K. Ballal, K. Wellnitz, D. Vela, M. Gong, R. Salazar, O. H. Frazier and H. Taegtmeyer (2009). "Markers of autophagy are downregulated in failing human heart after mechanical unloading." Circulation **120**(11 Suppl): S191-197.

Kawaguchi, T., G. Takemura, H. Kanamori, T. Takeyama, T. Watanabe, K. Morishita, A. Ogino, A. Tsujimoto, K. Goto, R. Maruyama, M. Kawasaki, A. Mikami, T. Fujiwara, H. Fujiwara and S. Minatoguchi (2012). "Prior starvation mitigates acute doxorubicin cardiotoxicity through restoration of autophagy in affected cardiomyocytes." Cardiovasc Res **96**(3): 456-465.

Khalfan, W. A. and D. J. Klionsky (2002). "Molecular machinery required for autophagy and the cytoplasm to vacuole targeting (Cvt) pathway in S. cerevisiae." Curr Opin Cell Biol **14**(4): 468-475.

Kirisako, T., Y. Ichimura, H. Okada, Y. Kabeya, N. Mizushima, T. Yoshimori, M. Ohsumi, T. Takao, T. Noda and Y. Ohsumi (2000). "The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway." J Cell Biol **151**(2): 263-276.

Kissova, I., M. Deffieu, S. Manon and N. Camougrand (2004). "Uth1p is involved in the autophagic degradation of mitochondria." J Biol Chem **279**(37): 39068-39074.

Klionsky, D. J., E. H. Baehrecke, J. H. Brumell, C. T. Chu, P. Codogno, A. M. Cuervo, J. Debnath, V. Deretic, Z. Elazar, E. L. Eskelinen, S. Finkbeiner, J. Fueyo-Margareto, D. Gewirtz, M. Jaattela, G. Kroemer, B. Levine, T. J. Melia, N. Mizushima, D. C. Rubinsztein, A. Simonsen, A. Thorburn, M. Thumm and S. A. Tooze (2011). "A comprehensive glossary of autophagy-related molecules and processes (2nd edition)." Autophagy **7**(11): 1273-1294.

Kobayashi, S., P. Volden, D. Timm, K. Mao, X. Xu and Q. Liang (2010). "Transcription factor GATA4 inhibits doxorubicin-induced autophagy and cardiomyocyte death." J Biol Chem **285**(1): 793-804.

Kostin, S., L. Pool, A. Elsasser, S. Hein, H. C. Drexler, E. Arnon, Y. Hayakawa, R. Zimmermann, E. Bauer, W. P. Klovekorn and J. Schaper (2003). "Myocytes die by multiple mechanisms in failing human hearts." Circ Res **92**(7): 715-724.

Kroemer, G., L. Galluzzi and C. Brenner (2007). "Mitochondrial membrane permeabilization in cell death." Physiol Rev **87**(1): 99-163.

Kubli, D. A. and A. B. Gustafsson (2012). "Mitochondria and mitophagy: the yin and yang of cell death control." Circ Res **111**(9): 1208-1221.

Kubli, D. A., X. Zhang, Y. Lee, R. A. Hanna, M. N. Quinsay, C. K. Nguyen, R. Jimenez, S. Petrosyan, A. N. Murphy and A. B. Gustafsson (2013). "Parkin protein deficiency

exacerbates cardiac injury and reduces survival following myocardial infarction." J Biol Chem **288**(2): 915-926.

Langston, J. W., P. Ballard, J. W. Tetrud and I. Irwin (1983). "Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis." Science **219**(4587): 979-980.

Laplante, M. and D. M. Sabatini (2012). "mTOR Signaling." Cold Spring Harb Perspect Biol **4**(2).

Lazarou, M., S. M. Jin, L. A. Kane and R. J. Youle (2012). "Role of PINK1 binding to the TOM complex and alternate intracellular membranes in recruitment and activation of the E3 ligase Parkin." Dev Cell **22**(2): 320-333.

Lee, J. Y., Y. Nagano, J. P. Taylor, K. L. Lim and T. P. Yao (2010). "Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy." J Cell Biol **189**(4): 671-679.

Linden, M., B. D. Nelson and J. F. Leterrier (1989). "The specific binding of the microtubule-associated protein 2 (MAP2) to the outer membrane of rat brain mitochondria." Biochem J **261**(1): 167-173.

Liu, L., D. Feng, G. Chen, M. Chen, Q. Zheng, P. Song, Q. Ma, C. Zhu, R. Wang, W. Qi, L. Huang, P. Xue, B. Li, X. Wang, H. Jin, J. Wang, F. Yang, P. Liu, Y. Zhu, S. Sui and Q. Chen (2012). "Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells." Nat Cell Biol **14**(2): 177-185.

Lu, L., W. Wu, J. Yan, X. Li, H. Yu and X. Yu (2009). "Adriamycin-induced autophagic cardiomyocyte death plays a pathogenic role in a rat model of heart failure." Int J Cardiol **134**(1): 82-90.

Lv, P., J. Huang, J. Yang, Y. Deng, J. Xu, X. Zhang, W. Li, H. Zhang and Y. Yang (2014). "Autophagy in muscle of glucose-infusion hyperglycemia rats and streptozotocin-induced hyperglycemia rats via selective activation of m-TOR or FoxO3." PLoS One **9**(2): e87254.

Maiuri, M. C., E. Zalckvar, A. Kimchi and G. Kroemer (2007). "Self-eating and self-killing: crosstalk between autophagy and apoptosis." Nat Rev Mol Cell Biol **8**(9): 741-752.

Mammucari, C., G. Milan, V. Romanello, E. Masiero, R. Rudolf, P. Del Piccolo, S. J. Burden, R. Di Lisi, C. Sandri, J. Zhao, A. L. Goldberg, S. Schiaffino and M. Sandri

(2007). "FoxO3 controls autophagy in skeletal muscle in vivo." Cell Metab **6**(6): 458-471.

Marcil, M., A. Ascah, J. Matas, S. Belanger, C. F. Deschepper and Y. Burelle (2006). "Compensated volume overload increases the vulnerability of heart mitochondria without affecting their functions in the absence of stress." J Mol Cell Cardiol **41**(6): 998-1009.

Nakai, A., O. Yamaguchi, T. Takeda, Y. Higuchi, S. Hikoso, M. Taniike, S. Omiya, I. Mizote, Y. Matsumura, M. Asahi, K. Nishida, M. Hori, N. Mizushima and K. Otsu (2007). "The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress." Nat Med **13**(5): 619-624.

Narendra, D., L. A. Kane, D. N. Hauser, I. M. Fearnley and R. J. Youle (2010). "p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both." Autophagy **6**(8): 1090-1106.

Narendra, D., A. Tanaka, D. F. Suen and R. J. Youle (2008). "Parkin is recruited selectively to impaired mitochondria and promotes their autophagy." J Cell Biol **183**(5): 795-803.

Narendra, D. P., S. M. Jin, A. Tanaka, D. F. Suen, C. A. Gautier, J. Shen, M. R. Cookson and R. J. Youle (2010). "PINK1 is selectively stabilized on impaired mitochondria to activate Parkin." PLoS Biol **8**(1): e1000298.

Novak, I., V. Kirkin, D. G. McEwan, J. Zhang, P. Wild, A. Rozenknop, V. Rogov, F. Lohr, D. Popovic, A. Occhipinti, A. S. Reichert, J. Terzic, V. Dotsch, P. A. Ney and I. Dikic (2010). "Nix is a selective autophagy receptor for mitochondrial clearance." EMBO Rep **11**(1): 45-51.

Okatsu, K., K. Saisho, M. Shimanuki, K. Nakada, H. Shitara, Y. S. Sou, M. Kimura, S. Sato, N. Hattori, M. Komatsu, K. Tanaka and N. Matsuda (2010). "p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria." Genes Cells **15**(8): 887-900.

Okamoto, K., N. Kondo-Okamoto and Y. Ohsumi (2009). "Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy." Dev Cell **17**(1): 87-97.

Palmeira, C. M., J. Serrano, D. W. Kuehl and K. B. Wallace (1997). "Preferential oxidation of cardiac mitochondrial DNA following acute intoxication with doxorubicin." Biochim Biophys Acta **1321**(2): 101-106.

Pankiv, S., T. H. Clausen, T. Lamark, A. Brech, J. A. Bruun, H. Outzen, A. Overvatn, G. Bjorkoy and T. Johansen (2007). "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy." J Biol Chem **282**(33): 24131-24145.

Pereira, G. C., et al. (2012). "Mitochondrionopathy phenotype in doxorubicin-treated Wistar rats depends on treatment protocol and is cardiac-specific." PLoS ONE **7**(6): e38867.

Picard, M., K. Csukly, M. E. Robillard, R. Godin, A. Ascah, C. Bourcier-Lucas and Y. Burelle (2008). "Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles." Am J Physiol Regul Integr Comp Physiol **295**(2): R659-668.

Piquereau, J., R. Godin, S. Deschenes, V. L. Bessi, M. Mofarrahi, S. N. Hussain and Y. Burelle (2013). "Protective role of PARK2/Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction." Autophagy **9**(11): 1837-1851.

Rodriguez, A., A. Duran, M. Selloum, M. F. Champy, F. J. Diez-Guerra, J. M. Flores, M. Serrano, J. Auwerx, M. T. Diaz-Meco and J. Moscat (2006). "Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62." Cell Metab **3**(3): 211-222.

Santos, D. L., A. J. Moreno, R. L. Leino, M. K. Froberg and K. B. Wallace (2002). "Carvedilol protects against doxorubicin-induced mitochondrial cardiomyopathy." Toxicol Appl Pharmacol **185**(3): 218-227.

Scarffe, L. A., D. A. Stevens, V. L. Dawson and T. M. Dawson (2014). "Parkin and PINK1: much more than mitophagy." Trends Neurosci **37**(6): 315-324.

Scherz-Shouval, R. and Z. Elazar (2011). "Regulation of autophagy by ROS: physiology and pathology." Trends Biochem Sci **36**(1): 30-38.

Serrano, J., C. M. Palmeira, D. W. Kuehl and K. B. Wallace (1999). "Cardioselective and cumulative oxidation of mitochondrial DNA following subchronic doxorubicin administration." Biochim Biophys Acta **1411**(1): 201-205.

Sha, D., L. S. Chin and L. Li (2010). "Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling." Hum Mol Genet **19**(2): 352-363.

Simonsen, A., R. C. Cumming, A. Brech, P. Isakson, D. R. Schubert and K. D. Finley (2008). "Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila." Autophagy **4**(2): 176-184.

Sishi, B. J., B. Loos, J. van Rooyen and A. M. Engelbrecht (2013). "Autophagy upregulation promotes survival and attenuates doxorubicin-induced cardiotoxicity." Biochem Pharmacol **85**(1): 124-134.

Smuder, A. J., A. N. Kavazis, K. Min and S. K. Powers (2013). "Doxorubicin-induced markers of myocardial autophagic signaling in sedentary and exercise trained animals." J Appl Physiol (1985) **115**(2): 176-185.

Solem, L. E., T. R. Henry and K. B. Wallace (1994). "Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration." Toxicol Appl Pharmacol **129**(2): 214-222.

Solem, L. E. and K. B. Wallace (1993). "Selective activation of the sodium-independent, cyclosporin A-sensitive calcium pore of cardiac mitochondria by doxorubicin." Toxicol Appl Pharmacol **121**(1): 50-57.

St-Pierre, J., J. A. Buckingham, S. J. Roebuck and M. D. Brand (2002). "Topology of superoxide production from different sites in the mitochondrial electron transport chain." J Biol Chem **277**(47): 44784-44790.

Suzuki, K., Y. Kubota, T. Sekito and Y. Ohsumi (2007). "Hierarchy of Atg proteins in pre-autophagosomal structure organization." Genes Cells **12**(2): 209-218.

Swain, S. M., F. S. Whaley and M. S. Ewer (2003). "Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials." Cancer **97**(11): 2869-2879.

Takeshige, K., M. Baba, S. Tsuboi, T. Noda and Y. Ohsumi (1992). "Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction." J Cell Biol **119**(2): 301-311.

Tanaka, A. (2010). "Parkin-mediated selective mitochondrial autophagy, mitophagy: Parkin purges damaged organelles from the vital mitochondrial network." FEBS Lett **584**(7): 1386-1392.
Tsukada, M. and Y. Ohsumi (1993). "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae." FEBS Lett **333**(1-2): 169-174.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." J Physiol **552**(Pt 2): 335-344.

Valente, E. M., S. Salvi, T. Ialongo, R. Marongiu, A. E. Elia, V. Caputo, L. Romito, A. Albanese, B. Dallapiccola and A. R. Bentivoglio (2004). "PINK1 mutations are associated with sporadic early-onset parkinsonism." Ann Neurol **56**(3): 336-341.

Valko, M., D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser (2007). "Free radicals and antioxidants in normal physiological functions and human disease." Int J Biochem Cell Biol **39**(1): 44-84.

Valko, M., C. J. Rhodes, J. Moncol, M. Izakovic and M. Mazur (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chem Biol Interact **160**(1): 1-40.

Vives-Bauza, C., R. L. de Vries, M. Tocilescu and S. Przedborski (2010). "PINK1/Parkin direct mitochondria to autophagy." Autophagy **6**(2): 315-316.

Xie, M., C. R. Morales, S. Lavandero and J. A. Hill (2011). "Tuning flux: autophagy as a target of heart disease therapy." Curr Opin Cardiol **26**(3): 216-222.

Xu, X., K. Chen, S. Kobayashi, D. Timm and Q. Liang (2012). "Resveratrol attenuates doxorubicin-induced cardiomyocyte death via inhibition of p70 S6 kinase 1-mediated autophagy." J Pharmacol Exp Ther **341**(1): 183-195.

Yen, H. C., T. D. Oberley, C. G. Gairola, L. I. Szweda and D. K. St Clair (1999). "Manganese superoxide dismutase protects mitochondrial complex I against adriamycin-induced cardiomyopathy in transgenic mice." Arch Biochem Biophys **362**(1): 59-66.

Yoshii, S. R., C. Kishi, N. Ishihara and N. Mizushima (2011). "Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane." J Biol Chem **286**(22): 19630-19640.

Zhang, H., M. Bosch-Marce, L. A. Shimoda, Y. S. Tan, J. H. Baek, J. B. Wesley, F. J. Gonzalez and G. L. Semenza (2008). "Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia." J Biol Chem **283**(16): 10892-10903.

Zhang, Y., H. El-Sikhry, K. R. Chaudhary, S. N. Batchu, A. Shayeganpour, T. O. Jukar, J. A. Bradbury, J. P. Graves, L. M. DeGraff, P. Myers, D. C. Rouse, J. Foley, A. Nyska, D. C. Zeldin and J. M. Seubert (2009). "Overexpression of CYP2J2 provides protection against doxorubicin-induced cardiotoxicity." Am J Physiol Heart Circ Physiol **297**(1): H37-46.

Zhang, Y. W., J. Shi, Y. J. Li and L. Wei (2009). "Cardiomyocyte death in doxorubicin-induced cardiotoxicity." Arch Immunol Ther Exp (Warsz) **57**(6): 435-445.

Zhao, T., X. Huang, L. Han, X. Wang, H. Cheng, Y. Zhao, Q. Chen, J. Chen, H. Cheng, R. Xiao and M. Zheng (2012). "Central role of mitofusin 2 in autophagosome-lysosome fusion in cardiomyocytes." J Biol Chem **287**(28): 23615-23625.

Zhou, S., C. M. Palmeira and K. B. Wallace (2001). "Doxorubicin-induced persistent oxidative stress to cardiac myocytes." Toxicol Lett **121**(3): 151-157.

Zhou, S., A. Starkov, M. K. Froberg, R. L. Leino and K. B. Wallace (2001). "Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin." Cancer Res **61**(2): 771-777.

Zoratti, M. and I. Szabo (1995). "The mitochondrial permeability transition." Biochim Biophys Acta **1241**(2): 139-176.