Molecular Regulation of Mitochondrial Dynamics in Anthracycline Cardiotoxicity

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Abstract

Doxorubicin (Dox) is a highly effective anti-tumor agent that is widely used to treat a variety of malignancies. However, its use is limited by its cardio-toxic effects which can induce heart failure. The underlying mechanism of Dox induced cardiotoxicity is not fully understood. The IKKβ -NF-κB signaling pathway regulates a variety of processes including inflammation, differentiation, and cell survival. Previous work from our lab established a critical survival role for IKKβ -NF-κB signaling in cardiomyocytes through suppressing mitochondrial perturbations induced by hypoxia. However, despite these findings, it remained undetermined whether the survival properties of IKKβ were restricted to hypoxia or extended more broadly to other cardiac abnormalities such as Dox cardiotoxicity. In this study we explore the role of IKKβ -NF-κB pathway in cardiac myocytes treated with Dox. Dox treatment caused critical mitochondrial defects including mPTP opening, loss of membrane potential ($\Delta\Psi m$), and ROS production. Levels of the mitochondrial fusion protein Mitofusin 2 (MFN2) were decreased in cardiac myocytes treated with DOX resulting in increased mitochondrial fission, loss of mitochondrial networks and impaired respiration. Interestingly, we identified that MFN2 degradation was through an autophagic process. Knockdown of ATG7 (a key regulator of autophagy) suppressed autophagy-mediated loss of MFN2 in cells treated with DOX. Markedly, wild type (IKKβ) but not a kinase inactive mutant ($IKK\beta_{k/m}$) suppressed autophagy mediated degradation of MFN2, and mitochondrial perturbation induced by Dox. Notably, restoration of mitochondrial fusion, oxidative respiration and cell viability by IKKβ was contingent upon the GTPase activity of MFN2. Importantly, we show that IKKβ directly forms a novel protein-protein complex with the outer mitochondrial membrane protein MFN2 which is critical for IKKβ mediated cardioprotection against doxorubicin cardiotoxicity. Hence, the findings of the present study

reveal a novel signaling axis that functionally couples innate signaling through IKKβ and MFN2 to mitochondrial fission and necrotic cell death during DOX cardiomyopathy.

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Dedication

I dedicate this thesis to my Zaida Ben Chochinov

“Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional.”

Brian Greene

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List of Abbreviations

$\Delta\Psi m$	Mitochondrial membrane potential
ATG	Autophagy related
ATG7	Autophagy related 7
ATP	Adenosine triphosphate
BAX	Bcl-2 associated X protein
Bcl-2	B-Cell lymphoma 2
BNIP3	Bcl-2 nineteen-Kilodalton interacting protein-3
Ca^{2+}	Calcium
CMT2A	Charcot-Marie-Tooth Disease 2A
c-Myc	Cellular Myc
COX1	Cytochrome c oxidase subunit 1
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DRP1	Dynamin-related protein 1
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAS	Tumor necrosis factor receptor superfamily member 6
FF	Form Factor
FIS1	Mitochondrial fission 1 protein
FZO	Fuzzy onions
GTP	Guanosine Triphosphate
HDAC	Histone Deacetylase
HEK 293	Human embryonic kidney 293 cells
HSP60	Heat shock protein 60
HR	Heptads repeat
IAP	Inhibitor of Apoptosis
IκBα	Nuclear factor kappa-B Inhibitor alpha
IKKα	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta

IKKγ	Inhibitor of nuclear factor kappa-B kinase subunit gamma
IMM	Inner mitochondrial membrane
IMS	Intermembranal space
IP	Immunoprecipitation
Mdivi	Mitochondrial division inhibitor 1
MFF	Mitochondrial fission factor
MFN1	Mitofusin-1
MFN2	Mitofusin-2
mPTP	Mitochondrial permeability transition pore
NDUFA9	NADH: Ubiquinone Oxidoreductase Subunit A9
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
p53	Tumor protein 53
p65	Nuclear factor NF-kappa-B p65 subunit
PINK1	PTEN-induced kinase 1
ROS	Reactive oxygen species
SERCA	Sarco/endoplasmic reticulum Ca^{2}-ATPase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOD	Superoxide dismutase
TLR2	Toll like receptor 2
TOM 20	Translocase of outer membrane receptor 20
Top II	Topoisomerase II
UCP3	Mitochondrial uncoupling protein 3
VDAC	Voltage-dependent anion channel

I. Introduction

Anthracyclines such as Doxorubicin (Dox) are highly effective chemotherapeutic drugs used to treat many forms of human cancer. It is however, well established that Dox treatment causes cardiotoxicity that can lead to heart failure.[1] Dox has been shown to affect several cellular events however the underlying mechanism of Dox cardiotoxicity has not been fully elucidated. Previous research has shown Dox toxicity to be associated with DNA damage, lipid peroxidation, iron metabolism, impaired calcium regulation, oxidative stress and impaired mitochondria and cell death[2–6].

Most studies have shown increased reactive oxygen species (ROS) production as the most prominent mechanism of Dox cardiotoxicity and that the mitochondria are the primary target of ROS[7]. The heart is abundant with mitochondria, which is responsible for many cellular processes including metabolism, respiration, calcium signaling and cell death. Various studies have shown increased ROS production due to Dox leads to mitochondrial DNA damage, impaired mitochondrial respiration, and mPTP opening[8].

Reactive oxygen species are unstable molecules that contain oxygen and when they build up in the cell can cause damage to RNA, DNA, and proteins that can lead to cell death. Mitochondrial ROS production has been linked to disruption of mitochondrial dynamics[9]. Mitochondrial dynamics is a homeostatic process that involves fission and fusion of mitochondria. Fusion proteins such as Optic atrophy 1 (OPA1), Mitofusin 1(MFN1) and Mitofusin 2 (MFN2) help mediate fusion of the inner and outer mitochondrial membranes, respectively. Mitochondrial fission is mediated by dynamin-related protein 1(DRP1). Maintaining an optimal balance of these two events is of vital importance for sustaining many mitochondrial processes like respiration, metabolism and even ROS production[10]. When the scale tips more to one side then

the other that's when we see mitochondrial dysfunction. Studies have shown that excess mitochondrial fusion can lead to enlarged mitochondria and dilated cardiomyopathy. Conversely, excessive mitochondrial fission can lead to fragmented mitochondria and increased ROS production[11, 12]. Therefore, balancing these processes is critical for optimal function of mitochondria. There is a growing body of evidence to suggest that proteins critical for regulating mitochondrial dynamics are disrupted by Dox treatment[13].

The NF-κB signaling pathway has been shown to be protective in the heart under hypoxic stress.[14] Indeed, studies from our lab have shown that this pathway elicits protective effects through activating pro- survival genes and inhibiting pro- death genes[15]. Earlier work from our lab also showed that the NF-κB signaling pathway can protect against hypoxia induced mitochondrial dysfunction [14]. One of the key regulators of the NF-κB signaling pathway in cardiac myocytes is IKKβ the catalytically active subunit of the IκB kinase complex (IKK). Our lab has identified IKKβ kinase activity to be important for NF-κB signaling and has a pro-survival role in cardiac cells conserving mitochondrial function[14, 16]. However, it remained undetermined whether IKKβ independent of its role in regulating NF-κB can regulate cell survival of cardiac myocytes.

In this study, we investigated this possibility and tested whether IKKβ could prevent mitochondrial perturbations and cell death of cardiac myocytes induced by Dox, with specific attention to the impact of IKKβ on mitochondrial dynamics. We provide new compelling evidence thatIKKβ protects against Dox cardiotoxicity by preventing autophagic removal of the mitochondrial fusion protein MFN2. More specifically we showed that IKKβ by preventing the autophagic removal of MFN2 prevents mitochondrial fission and cell death of cardiac myocytes

induced by Dox. Our findings demonstrate for the first time that IKKβ suppresses cell death of cardiac myocytes by suppressing mitochondrial perturbations induced by Dox.

II. Literature Review

1.0 Doxorubicin

Doxorubicin (Dox) is highly effective anticancer agent of the Anthracycline family of drugs. Dox was first isolated from colonies of a pigment producing soil bacterium *streptomyces peucetius casesius* in the 1960's [17]. Dox is able to treat a variety of adult and childhood malignancies such as breast cancer, small cell lung cancer, Hodgkin and non-Hodgkin lymphoma[18]. Despite Dox efficient use to treat cancer its risk of inducing acute and/or chronic cardiac toxicity including contractile failure, mitochondrial dysfunction, increased reactive oxygen species production and necrotic cell death are limiting its use[1]. Efforts to combat Dox toxicity would keep it as a preferred drug for treating cancer patients and for that we must have a clear understanding of its mechanism.

1.1 Pharmacokinetics and Pharmacodynamics of Dox

Dox is administered through intravenous injection (i.v.). Dosing is most commonly 60-75mg/m^2 given at21-day intervals but also a weekly dose of 20mg/m^2 has also been utilized[19]. Once in the plasma Dox has a distributive half-life of ~5 min and is rapidly taken up by tissues. Dox is able to enter cells passively through its lipophilicity allowing it to cross the lipid bilayer[20]. While its distribution and uptake are quite quick its elimination has been reported to be around 20-48 hours most likely due to its high affinity to binding to tissue. Dox is able to be metabolized through a few different routes but about half is eliminated from the body unaffected[21]. The primary pathway involves a reduction of a ketone group to a hydroxyl group forming a less active metabolite doxorubicinol[22,23]. Other ways to metabolize is to utilize mitochondrial enzymes(NADH Dehydrogenase) to cause a reduction of Dox and form a semiquinone free radical or deglycosidation[24]. Once inside the cell Dox has can utilize its anti-cancer properties.

Dox will intercalate into the DNA between adjacent base pairs of the double helix and causes DNA strand breakage and also inhibits topoisomerase II (TopII) [25]. TopII is an enzyme that is important in DNA replication and transcription [26]. Since cancer cells have much higher rates of division they have elevated levels of topoisomerase II[27]. Dox effect on TopII as well as DNA strand breakage will eventually lead to inhibition of DNA replication, protein synthesis and ultimately cell death[28]. Studies have also suggested that Dox redox cycling from its quinone to its semiquinone structure lead to excessive ROS production[5].

1.2 Cardiotoxicity

In 3 randomized phase III studies researchers found that Dox related congestive heart failure (CHF) occurred in a dose dependent manner[29]. A steady increase of participants developed signs of DOX induced CHF when the cumulative dosage of Dox exceeded 550mg/m^2, not only that CHF occurred with greater frequency and at a lower cumulative dose than was previously stated[29]. Incidence of Dox cardiotoxicity can manifest as both acute and chronic toxic effects. Acute cardiotoxicity can manifest as arrhythmias or hypotension and occurs within a few days of Dox treatment [30,31]. This is often reversible, however it usually because treatment was terminated. The full mechanism of Dox cardiotoxic effects still requires to be fully explored but is most likely multifactorial.

1.2.1 DNA Damage

As previously mentioned, Dox affects DNA replication within cancer cells specifically through inhibition of TopII. Within the heart there are two types of topoisomerase TopIIa and TopIIb, Whereas TopIIa is mostly found in proliferating cells[27], TopIIb is found in most cell types and is the predominate isoform in cardiomyocytes [32]. Like in cancer cells with TopIIa, Dox will also target TopIIb. This creates a complex between Dox, TopIIb and DNA causing double stranded

breaks and can lead to cell death. Studies have proven that TopIIb is involved in the progression of Dox cardiotoxicity through knockout experiments. Mice that had cardiomyocyte specific deletion of TopIIb were protected from cardiac damage due to Dox[7].

1.2.1 Calcium Dysfunction

Calcium homeostasis is an important part of cardiomyocyte function and contractility. Research has established dysregulated calcium homeostasis as one of the hallmarks of Dox cardiotoxicity. Dox cardiotoxicity has been shown to affect the sarcoplasmic reticulum and its ability to sequester calcium within it through modulation of the sarco/endoplasmic reticulum Ca^2-ATPase (SERCA)[33,34]. Dox has also been shown to be involved in inhibiting calcium uptake to the sarcolemma through inhibiting the sodium calcium exchanger[35]. Furthermore Dox limits calcium storage within the mitochondria through alterations of specific calcium channels causing mitochondrial dysfunction leading to excessive cytoplasmic calcium release and cell death[36].

1.2.2 Iron metabolism

It has been noted that Dox has a strong affinity for iron, forming complexes that may react with oxygen and cause an increase in ROS[37,38]. Dox has also been shown to interfere with iron metabolism through inhibiting translation of iron sequestering proteins [39]. Dox can also cause iron accumulation within the mitochondria[40]. Preventing this accumulation either through maintaining mitochondrial iron transport proteins or by use of the iron chelator Dexrazoxane protected against Dox induced cardiac damage[40]. Although the function of iron in Dox cardiotoxicity has yet to be fully elucidated it is clear that it is has a critical role in its progression.

1.2.3 Oxidative Stress/ROS Production

Studies have shown that an increase in ROS leads to oxidative stress and a reduction in antioxidant levels as a underlying cause of cardiac dysfunction.[41] Mitochondrial defects such as disruption of respiration, mPTP opening, ROS and alteration in the mitochondrial dynamics are implicated in Dox cardiotoxicity. The following section, details how Dox treatment influences mitochondrial morphology and function; moreover, how cardiomyocytes with defective mitochondria undergo cell death. The mode of Dox mediated cell death is also controversial. Previously when our understanding of cell death pathways was limited, Dox was considered to strictly cause apoptotic cell death. With the recent advances in the field, more evidence suggests Doxorubicin mediated cell death to be more complex.

1.3 Dox Induced Cell Death

Dox can elicit cardiac cell death through apoptosis, necrosis or autophagy.[42] Interestingly mitochondria have been implicated in these cellular processes and therefore are considered to play an important role in determining the fate of the cell.[43] Different outcomes with respect to modes of cell death can be attributed to different models, doses and timing of delivery of Dox. Even though each death pathway has its own distinct features, the crosstalk between these pathways has been reported.[44] The different aspects of each death pathway are described below.

1.3.1 Apoptosis

Apoptosis is one of the most well-established cell death pathways. It is clearly identifiable from its associated proteins in either its intrinsic or extrinsic pathway. It also has clearly defined morphological features such as membrane blebbing, chromosome condensation and apoptotic bodies[45]. Many studies have shown that Dox exposure can induce both intrinsic and extrinsic cell death pathways in cardiomyocytes[46]. Extrinsically Dox has been shown to activate pro-apoptotic

genes such as Fas, c-Myc, p53 and Toll-Like Receptor 2 (TLR2) [47–49]. Intrinsically Dox has been shown to associate with the mitochondria by activating key pro-apoptotic genes like Bax and inducing caspase-3 activation[36, 37].

1.3.2 Autophagy

Autophagy is a highly conserved process and is considered as a protective mechanism. Autophagy is characterized by damaged organelles, misfolded proteins or macromolecules that are sequestered by a doubled-membrane vesicle, called an autophagosome. The autophagosome then migrates and fuses to hydrolytic lysosomes forming autolysosomes for subsequent degradation and/or recycling. [52] Autophagy is largely controlled by autophagy related (ATG) genes and occurs through a series of steps including, initiation, nucleation, elongation, maturation and finally degradation. Each of the steps is orchestrated by a group of proteins that help regulate the process of autophagy. There is a delicate balance in maintaining homeostatic levels of autophagy. When the scale tips in favour of too much or too little autophagy the effects can be detrimental [53] This is evident in cardiomyocyte impairment observed, when Dox induced cell stress causes excessive removal of damaged mitochondria (too much autophagy) or if the autophagic pathway is impaired resulting in the accumulation of damaged mitochondria (too little autophagy).[54,55] Studies from our lab have linked Dox with an induction of autophagy through an increase number of autophagosomes.[6]

1.3.3 Necrosis

Unlike apoptosis, necrosis has been established to be a less orderly form of cell death. Necrosis has been shown to occur during times of low ATP[56]. Necrosis can be characterized by a few key distinct features such as cellular swelling, organelle disruptions and subsequent inflammatory response due to cellular contents being extruded into extracellular space.[44,57] Importantly Dox

treatment can cause calcium overload in the mitochondria leading to increased oxidative stress, increased mitochondrial damage and pore opening all of which ultimately leads to necrotic cell death.[6] More importantly, previous work from our lab has identified Bnip3; a key mitochondrial death protein to be involved in the Dox induced necrotic cell death of cardiomyocytes.[6] Our finding suggests a clear link between mitochondrial defects and necrotic cell death of Dox treated cardiomyocytes.

2.0 Dox and mitochondrial dysfunction

The mitochondria are of vital importance for energy production and myocyte viability. Research showed that Dox accumulates in the mitochondria of heart tissue at a much higher rate than plasma [58]. The mitochondrial accumulation of Dox can cause varying issues to the mitochondria including respiratory chain defects [59,60] mitochondrial transition pore (mPTP) opening[6],loss of mitochondrial membrane potential ($\Delta\Psi m$)[6]increase in fission proteins[6,54] and ultimately cell death[61,62]. Studies from our lab have shown Dox treatment leads to disruption of the protein complex formed by Uncoupling Protein 3 (UCP3) and Cytochrome c Oxidase subunit 1 (COX1) resulting into impaired respiration, increased ROS production and cell death[6]. Interestingly Dox treatment influences mitochondrial morphology as well as its function to perform oxidative metabolism. However, at this point it is unclear whether mitochondrial morphology is altered in Dox first or as a consequence of ROS and impaired mitochondrial respiration. Hence, determining whether an alteration in mitochondrial morphology underlies defects in mitochondrial respiration will also be examined in this study. To provide a better understanding of the processes that govern mitochondrial morphology we will look into the key proteins of mitochondrial dynamics described below.

3.0 Mitochondrial Dynamics

The heart is enriched with mitochondria, making up about 30% of the cardiomyocyte[63]. Structurally, mitochondria are membrane organelles that consist of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) which are separated by an inner membrane space (IMS). The inner membrane is comprised of many cristae which is the site for electron transport. A key feature of the mitochondrion is its ability to produce energy in the form of ATP through respiration, which depends on the electron transfer between respiratory complexes (ETC), located on the cristae of IMM. Mitochondria are not only an energy source but are also involved in metabolic activities, calcium regulation, regulation of oxidative stress and cell death[64–67]. Defects in the mitochondria can lead to extensive and irreparable injury to the heart[12, 55].

3.1Background

Initially mitochondria were thought to be static and linked to energy production, however through live imaging studies, it is now well established that these structures are dynamic and move freely throughout the cell[69]. These fundamental observations led to several more discoveries regarding mitochondrial function, such as ER tethering and its effect on cell death pathways [70,71]. How the mitochondria move, and remodel is through an intricate dance of fission and fusion events. These processes are integral for maintaining normal cell growth and can be adjusted to meet energy demand. Disruption of balance between mitochondrial fission and fusion processes can lead to cardiac damage[72–75].

3.2Mitochondrial Fission

Mitochondrial fission is the process of division through highly enzymatic GTPase proteins such as DRP1 (dynamin-related protein 1), FIS1 (mitochondrial fission protein 1) and MFF

(mitochondrial fission factor) with DRP1 playing a central role in the process. Regulation of mitochondrial fission and how the fission of the IMM starts is complex and are less known. The process begins with DRP1 migrating from the cytosol and localizing to the OMM where it interacts with FIS1 at specific fission sites[76]. More recent studies have shown MFF to be a DRP1 receptor at the mitochondria [77]. Once translocated DRP1 is able to form a ring around the mitochondrion where it constricts and eventually severs the mitochondrion in two.[78] The process of fission is necessary for a range of cellular functions such as segregating damaged mitochondria for mitochondrial specific autophagy (mitophagy)[79], mitochondrial redistribution[80], and Cytochrome c release during apoptosis[81].

3.2.1 Mitochondrial Fission in the Heart

In terms of the importance of mitochondrial fission in the heart, knockout/knockdown studies of DRP1 have been shown to induce mPTP opening, decreased mitochondrial clearance and cause cardiac hypertrophy[82] . These studies demonstrate the importance of mitochondrial fission in the heart. Cardiovascular disease models such as cardiac hypertrophy and ischemia/reperfusion have shown increased DRP1mitochondrial translocation leading to increased mitochondrial fragmentation, increased ROS and overall mitochondrial dysfunction[70, 71]. These findings suggest that excessive mitochondrial fission can be detrimental. Furthermore, inhibition of DRP1 either through pharmacological agents (Mdivi) or siRNA targeted against DRP1were able to diminish signs of cardiac dysfunction. More importantly they were able to mitigate excessive DRP1 mitochondrial translocation thereby maintaining mitochondrial networking[71, 72].

3.2.2 Mitochondrial Fission and Dox

Much like heart failure and ischemia reperfusion models mitochondrial fission is upregulated in Dox[73, 74]. Previous studies from our lab indicated that Dox has an effect on mitochondrial

homeostasis. Dox treatment of cardiomyocytes increased mitochondrial targeting of DRP1and induced mitochondrial fragmentation[61]. These dynamic impairments led to mitochondrial dysfunction through increased ROS, mPTP opening and subsequent cardiac cell death. Our lab and others have shown that Bnip3 a known inducer of cardiac cell death by Dox[6] leads to DRP1 translocation and increased mitochondrial fission[88].

3.3 Mitochondrial Fusion

Similar to fission, mitochondrial fusion also utilizes GTPase proteins to perform its function. On the OMM there are two main proteins Mitofusin-1 (MFN1) and Mitofusin-2 (MFN2) and at the IMM is Optic atrophy-1 (OPA1) [89]. Fusion occurs when two distinct mitochondria are tethered together by interaction between Mitofusin proteins, MFN1 and MFN2 on the OMM. Studies have shown that OPA1 undergoes processing and cleaved into several different isoforms with each on with a particular role in the fusion of the IMM[90]. Fusion regulates and effects a multitude of mitochondrial functions including mitochondrial metabolism and respiration[91,92] , mitochondrial membrane potential ($\Delta\Psi m$)[93] and cell death[94]. Importance of mitochondrial fusion proteins is highlighted in studies where whole-body knockouts of MFN1, MFN2 and OPA1genes have been shown to be embryonically lethal[95,96].

3.3.1 Mitochondrial fusion and the Heart

In the context of the heart, mice lacking both MFN1 and MFN2 (DKO) through genetic inactivation mid-gestation led to impaired mitochondrial biogenesis and mitochondrial turnover in the postnatal period[97]. Moreover these mice eventually developed cardiomyopathy and none survived past 16 days of age[97]. Genetic ablation of MFN1 and MFN2 during adulthood showed no incidence of cardiomyopathy and even protection against Ischemia/Reperfusion injury[98]. However these hearts had atypical mitochondrial morphology, impaired mitochondrial

respiration as well as weakened contractile function[98]. This demonstrated that these mice did not have healthy mitochondria. Murine models looking at partial ablation of OPA1 in the heart also show reduced cardiac output[99]. Further studies looking at mitochondrial dynamics and cardiovascular disease purport that the fusion proteins OPA1 and MFN2 decrease under cardiac injury[100,101]. These studies showcase the importance of mitochondrial fusion in cardiac development and maintaining cardiac function. It has been well established that mitochondrial fusion can be utilized for cardioprotection. HL-1 cardiac cell lines subjected to simulated ischemic reperfusion injury (IRI) were rescued from cardiac injury (mPTP opening and cell death) when over-expressed with MFN1 or MFN2[84]. Disproportioned mitochondrial dynamics has been shown in the development of diabetic cardiomyopathy (DCM) in mice[102]. Treating the affected mice with MFN2 expressing adenovirus were able to prevent excessive oxidative stress, restore some respiratory capacity and improve overall mitochondrial function[102]. As a result, the incidents of impaired mitochondrial respiration, where Mitofusin proteins are either ablated or down regulated suggest that the fusion process is critical and a deciding factor for mitochondrial respiration and bioenergetics. Based on these findings it's clear that mitochondrial fusion, but more specifically, MFN2 plays a vital role in the safeguarding of cardiomyocyte health.

4.0 Mitofusin 2

Mitofusin 2 is the human homolog of fuzzy onions protein (Fzo) initially characterized in *Drosophila*[103]. It is a 757 amino acid protein that spans the OMM. It has a few conserved domains including a large cytosolic N-terminal GTPase domain, two coiled-coil domains (HR1 and HR2), and a transmembrane domain. The second HR domain allows for the tethering of neighbouring mitochondria, these dimerization can be between MFN2 alone or between MFN1 and MFN2[104]. When the GTPase domain is hydrolysed it allows for the mitochondrial fusion

reaction[103]. Although MFN1 and MFN2 share very similar structural characteristics they differ greatly in their functional capabilities

4.1 Mitofusin 2 Function

MFN2 is well established for its role in fusion, however, recent research has revealed that it is involved with many other cellular functions independent of its fusogenic properties. Still focusing on the mitochondria, research has demonstrated MFN2's involvement in mitochondrial metabolism. Studies indicated that when MFN2 is repressed there was a reduction in glucose oxidation and oxygen consumption[105]. Along with reduced respiratory function they reported a decline in mitochondrial membrane potential, signifying MFN2's importance in maintaining mitochondrial integrity.[105] Research that looked at recapitulating MFN2 in muscle cells saw increases glucose oxidation as well as improved mitochondrial membrane potential[106]. Moving outside the mitochondria studies have identified MFN2 on the endoplasmic reticulum (ER) where it aids in establishment of ER-mitochondrial contacts. These ER-mitochondrial contacts allow for efficient calcium (Ca^{2+}) transport from ER to the mitochondria[107]. MFN2 ablation studies confirmed this phenomenon by showing, in the absence of MFN2, there is greater ER-Mitochondria distance and reduced ER Ca^{2+} transport[108]. In the last few years MFN2 has been reported to be associated with mitophagy[109]. Mitophagy is a process for the selective removal of damaged mitochondria through specialized autophagic clearance. The hallmark proteins involved in mitophagy are PINK1 a serine /threonine protein kinase and Parkin an E3 ubiquitin ligase[110]. Upon stress, PINK1 is translocated to depolarized mitochondria where it recruits Parkin where it can ubiquitinate OMM proteins[111]. MFN2 has been reported to be phosphorylated by PINK1 and in turn allows recruitment of Parkin where it can ubiquitinate MFN2 leading to targeting of mitochondria for degradation[112]. Studies examining MFN2 deletion in cardiomyocytes revealed

increased accumulation of autophagosomes showcasing the importance of MFN2 in the mitophagy/ autophagic degradation process[113].

4.2 Mitofusin 2 in Disease

Given the importance of maintaining mitochondrial dynamics it is no surprise that MFN2 plays a role in the progression and development of many diseases and disorders. Several neurological conditions have been linked to alterations in MFN2 expression including Alzheimer's[114 115], Parkinson's[116] and Charcot Marie Tooth Disease type 2A (CMT2A)[117]. Furthermore looking into skeletal muscle and liver tissue in a rodent obesity and type II diabetes model showed significant down regulation of MFN2 leading to mitochondrial perturbations and ultimately insulin resistance[118] . It was previously mentioned the effects of disrupted mitochondrial fusion specifically with regard to MFN2 in the progression of heart disease, however, MFN2 regulation and response in the context of Dox toxicity have only recently begun to be investigated. A study by Tang et al has recently shown MFN2 down regulation in cardiomyocytes treated with Dox[119]. However, the mechanism as to how Dox influences MFN2 is still unclear.

5.0 IKKβ-NF-κB Signaling Pathway

NF-κB signaling is a well-known pro inflammatory response pathway[120]. Transcription factor nuclear factor kappa B (NF-κB) is activated through the degradation of its inhibitor IκBα. There are two distinct signaling pathways for NF-κB activation the canonical and non-canonical pathways. Under basal conditions in the canonical pathway NF-κB is present as an inactive dimer in the cytoplasm. NF-κB can be comprised of 5 different protein subunits however the most well characterized dimer in the canonical signalling pathway contains the p50 and p65 subunits [121]. The NF-κB inactive dimer is bound to its inhibitor protein IκBα within the cytoplasm[122]. IκBα binding to NF-κB masks its nuclear localization sequence of the p65 subunit

preventing its nuclear translocation[123]. NF-κB activation and its subsequent nuclear translocation can only occur following phosphorylation and degradation of IκBα by IKK kinase [124]. IκBα phosphorylation is mediated by the IκBα kinase (IKK) complex which consists of three subunits IKKα, IKKβ and IKKγ (NEMO) [125,126]. IKKβ is the functional unit of this complex and it essential in the phosphorylation of IκBα at serines 32 and 36 which allow for its ubiquitination and subsequent proteasomal degradation[127,128]. Once NF-κB is free of IκBα it then can translocate to the nucleus. Knock- out and loss of function studies of IKKβ have proven it to be the key subunit within the IKK complex of the canonical pathway[129,130]. Previously in our lab we have shown NF-κB to be cytoprotective in ventricular myocytes in response to hypoxic stress. [14]

5.1 IKKβ-NF-κB Role in Cell Death/ Cell Survival

Research from our lab has shown a link between the death gene Bnip3 and the transcription factor nuclear factor kappa B (NF-κB). NF-κB has been shown to transcriptionally silence Bnip3 gene expression through interactions with HDAC[131]. Silencing of Bnip3 expression through NF-κB is important in preventing cardiac cell death. IKKβ is the functionally active subunit of the IKK complex and is vital for activating NF-κB[16]. Once activated NF-κB has been shown to regulate many survival proteins like anti-apoptotic Bcl-2 family members, IAPs and SOD[132–134]. Hypoxic stress studies performed on cultured cardiomyocytes revealed that overexpression of IKKβ can ameliorate mitochondrial defects and cardiac cell death[14].

5.2 IKKβ-NF-κB in Preventing Dox Induced Mitochondrial Perturbations

Most recently our lab has shown the importance of NF-κB pathway in preventing Dox- induced cardiac cell death by a mechanism that impinges on e mitochondria[135]. Our studies revealed that

the NF-κB signaling pathway is impaired when cells are treated with Dox and this subsequently led to permeability transition pore (mPTP) opening and eventual cell death[135]. Since our recent studies demonstrate that restoration of NF-κB signaling by expressing IKKβ was sufficient in preventing mitochondrial defects of Dox treated cardiomyocytes. Therefore, in this study we investigated whether the cytoprotective properties conferred by IKKβ are operationally linked to mitochondrial dynamics in cardiac myocytes treated with Dox.

III. Rationale and Hypothesis

Rationale: It is well established that ROS is one of main causes of Dox induced cardiac cell death. ROS production in Dox treated cardiomyocytes is attributed to mitochondria dysfunction. Previous work from our lab showed a critical survival role for IKKβ -NF-κB signaling pathway in cardiomyocytes for suppressing mitochondrial dysfunction induced by hypoxia. Whether the IKKβ -NF-κB signaling pathway exerts a cardioprotective effect in Dox induced cardiotoxicity has not been established. Therefore, we investigated the effects of IKKβ -NF-κB signaling on mitochondrial and cellular function in Dox treated ventricular cardiac myocytes.

Hypothesis: IKKβ will protect against Dox cardiotoxicity through preserving mitochondrial function and reducing mitochondrial damage and therefore will minimize cardiac cell death and improve cardiac function.

IV. Materials and Methods

1. Neonatal cardiomyocyte Isolation and Cell Culture:

Neonatal ventricular myocytes were isolated from 1-2-day-old Sprague-Dawley rat pups. Pups were sacrificed by cervical dislocation. Myocytes were plated at a density of 1 x 10^6 cells per 35mm plate or $3.2x10^5$ per coverslip. Cells we incubated overnight at 37^oC in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) supplemented with 17mM HEPES, 3mM NaHCO3, 2mM L-glutamine, 50µg/mL gentamicin and 10% fetal bovine serum (FBS), then the myocytes were transferred to DF serum-free (DFSF) media for 24hrs. For this study cells were treated with DOX (1µM for 24hr, Pfizer)

Human embryonic kidney (HEK) 293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 0.1mM MEM Non-Essential Amino Acids (NEAA), 2mM L-glutamine and 1% Pen-Strep as previously reported. Cells were seeded at a density of 2-5x 10^4 viable cells/cm^2 to allow for 70-80% confluency.

Cultured cells (HEK293) transfected with expression plasmids using Effectene reagent (Qiagen, Inc.) or infected with adenovirus carrying the desired gene 24-hr after plating under DFSF conditions.

2. Plasmids, shRNAs and Adenoviruses

Plasmids and adenovirus encoding wild type IKKβ (pCR-Flag-IKKbeta Addgene plasmid # 15465) the kinase active form of IKKβ and $IKKβ_{k/m}$ (pCR-Flag-IKKbeta-KM Addgene plasmid# 15466) a kinase deficient mutant with a Lysine to Alanine substitution at position 44 were generated. Plasmids and adenovirus encoding wild type MFN2 (MFN2 wt), a GTPase defective mutant MFN2 (MFN2aa) and ATG7 shRNA were also used for this study. Replication defective adenoviruses were propagated in HEK293 cells and harvested by freeze-thawing. Adenoviral

titers were determined by plaque assay in HEK293 cells. PCDNA-HA-FLAG plasmid served as control for studies with plasmids and empty cytomegalovirus (CMV) adenovirus served as control for our studies with virus. Cells were transfected for 24-hrs at a multiplicity of infection (MOI) of 10-25 to achieve gene delivery to ≥ 90% of myocytes. After 24-hrs cardiomyocytes were then treated with Dox.

3. Cell Viability Assay:

Cell viability was analyzed through epifluorescent microscopy. Myocytes were stained with 2 µM calcein acetoxymethylester (Calcein-AM, Invitrogen) to visualize green cells (live) and 2 µM ethidium homodimer-1 (VWR) to visualize red cells (dead). After treatment, cells on coverslips were washed with PBS and then incubated for 30min at 37°C with DFSF media containing both Calcein-AM and Ethidium homodimer-1. After incubation the coverslips were inverted onto glass slides and visualized using Olympus AX-70 research fluorescence microscope at x200 magnification. Image J software was used to quantify images from the microscope. A total of three independent myocyte isolations were tested and at least 200 cells for each condition were analyzed.

4. Mitochondrial health and functional Assays

4.1 Reactive Oxygen Species Assay

To observe ROS production, vehicle and Dox treated cells were treated with fresh DFSF media with 2.5 µM of Dihydroethidium (Molecular Probes) added and then kept for 30 minutes at 37°C in the incubator. Cells were visualized by epifluorescence microscopy using Olympus AX-70 fluorescence microscope. The increased intensity of red dye signifies an increase in ROS production.

4.2 Mitochondrial Membrane Potential (ΔΨm)

Mitochondrial membrane potential (ΔΨm) in cells was assessed by epifluorescence microscopy. Vehicle and Dox treated cells were pre-incubated with 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM, Molecular Probes). Cells were mounted with florescent reagent and examined with an Olympus AX-70 fluorescence microscope. Images were quantified using Image J software. Proper functioning mitochondria within cardiomyocytes showed a bright red stain. Conversely, red stain becomes subtle when mitochondrial membrane potential dissipates.

4.3 mPTP Opening Assay

To analyze mitochondrial permeability transition pore (mPTP) opening, cells were treated with 5μM calcein-AM (Molecular probes) and Cobalt Chloride (5 mmol/L) and then incubated for 30 min at 37° C. Cells were mounted with fluorescent reagent and examined with Olympus AX-70 research epifluorescence microscope at 600x magnification. The formation of mPTP opening was observed as a reduction in the intensity of mitochondrial calcein staining. Image J software was used to measure the individual cell's fluorescence intensity of calcein-AM (green).

4.4 Mitochondrial Fragmentation

Mitochondrial morphology was assessed through immunofluorescent staining. Cardiac myocytes were fixed with ice cold ethanol (70%) and immunostained for mitochondrial protein Hsp60 (Santa Cruz # sc-13966). Images were acquired using research fluorescence microscope (AX10 observer SD) under the magnification of 630 x at room temperature using ZEN software (Zeiss). Using Image J software, raw images were binarized and mitochondria were subjected to particle analysis to obtain values for form factor (FF: perimeter2/4π·Area) which is a measure for both length and width. Minimal value of 1 indicates circularity, an increase in the values indicate elongation and branching.

4.5Mitochondrial Respiration:

Cardiomyocytes were cultured in 96-well plates and treated with either IKKβ, $IKK\beta_{K\text{-}M}$ or MFN2aa adenovirus followed by 24 hours of Dox treatment. The plate was assessed for mitochondrial oxygen consumption rate (OCR), using XFe96 Cell Mito Stress Analyzer (Agilent). In brief, culture medium was changed 1 hour before the assay to XF Base medium (Agilent 102353-100), supplemented with 1 mM Pyruvate (Gibco), and 10 mM D-glucose then pH to 7.4. Cells were then incubated at $37^{o}C$. Cells were then injected sequentially through ports in the Seahorse Flux Pak cartridges with oligomycin (1 μM), FCCP (2-[2-[4-(trifluoromethoxy) phenyl] hydrazinylidene]-propanedinitrile) (1 μM), and rotenone (1 μM) and antimycin (1 μM). Respiration data was analyzed using the Seahorse XF Cell Mito Stress Test Report Generator software. OCR, maximal respiration, and spare respiratory capacity were determined.

5. Western Blot Analysis and Immunoprecipitation

Neonatal myocytes were harvested in RIPA lysis (1.0% deoxycholate, 140 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with the addition of protease inhibitors (0μl of NaF, 10μl of PI, 10μl of Na3VO4, and 3.4μl PMSF per 1mL of RIPA). Lysate was then measured for protein concentration through bicinchoninic acid (BCA). Protein cell lysate (20-25 μg) were then denatured for 5 minutes at $100^{O}C$ and resolved on a denaturating sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel at 80 volts for 20 minutes followed by 100 volts for ~1 hour. The protein lysate was transferred to a nitrocellulose membrane at 100 volts for one hour at $4^{O}C$. Membranes were subjected to Ponceau staining to visualize protein bands and blocked for 1 hr in 5% skim milk in TBS-T (50mM Tris-HCl, 150mM NaCl, 0.3% Tween- 20, pH 7.4) at room temperature. Membranes were incubated with primary IgG antibodies directed against IKKβ (NEB#2678), NF-κB p65 (NEB#8242), MFN2 (NEB#9482), ATG7 (NEB#2631), p-DRP1

(s616) (NEB#3455), DRP1 (NEB#8570), VDAC (NEB#4661), TOM 20 (sc-17764), NDUFA9 (#459100), Actin (Sigma #A2172-.2ML) at 1:1000 dilution overnight at 4 C. Following incubation, membranes were washed three times with 1x TBS-T for 10 mins each and incubated with specific secondary antibodies for 1 hr at room temperature. Chemiluminescence reaction using horseradishes peroxidase (HRP) conjugated antibody with enhanced chemiluminescence (ECL) reagents (GE Healthcare) was used to detect bound proteins. Membranes were visualized using Amersham Imager 680 (GE Healthcare). For immunoprecipitation (IP), lysate derived cardiomyocytes (1000µg protein, lysed with RIPA buffer) was immunoprecipitated with murine Mitofusin 2 antibody (Abcam# Ab56889, 1 µg antibody/150 µg of protein) or IgG2a (Control for Mitofusin 2 antibody) using Dynabeads™ Protein G IP Kit (Thermo Fisher Scientific) and was probed with antibody against rabbit IKKβ antibody.

6. Quantitative real-time PCR (qPCR)

RNA extractions from cardiomyocyte were isolated using GenElute Mammalian Total RNA kit (Sigma-Aldrich). Primers were designed to detect MFN2 mRNA forward 5'-TCAATGGCATCTTTGAGCAG-3', reverse 5'-CAATCCCAGATGGCAGAACT-3' and L32 mRNA as a house-keeping control gene: forward 5'-TAAGCGAAACTGGCGGAAAC-3', reverse 5'-GCTGCTCTTTCTACGATGGCTT-3' (Sigma, Accession number; XO 6483). qPCR was performed using CXF96 qPCR detection system (Bio-Rad) fold change in gene expression was calculated by ΔΔCT method.

7. Mass Spectroscopy

For mass spectroscopy lysate derived from HEK 293 cells over expressing MFN2 and either IKKβ or $IKK\beta_{k/m}$ as well as control (PCDNA-HA-Flag) was immunoprecipitated with murine Mitofusin 2 antibody or IgG2a using Dynabeads™ Protein G IP Kit. Samples were then stored in

100mM Tris buffer (pH 8.5) and sent to Manitoba Centre for Proteomics and Systems Biology for processing.

8. Statistical Analysis

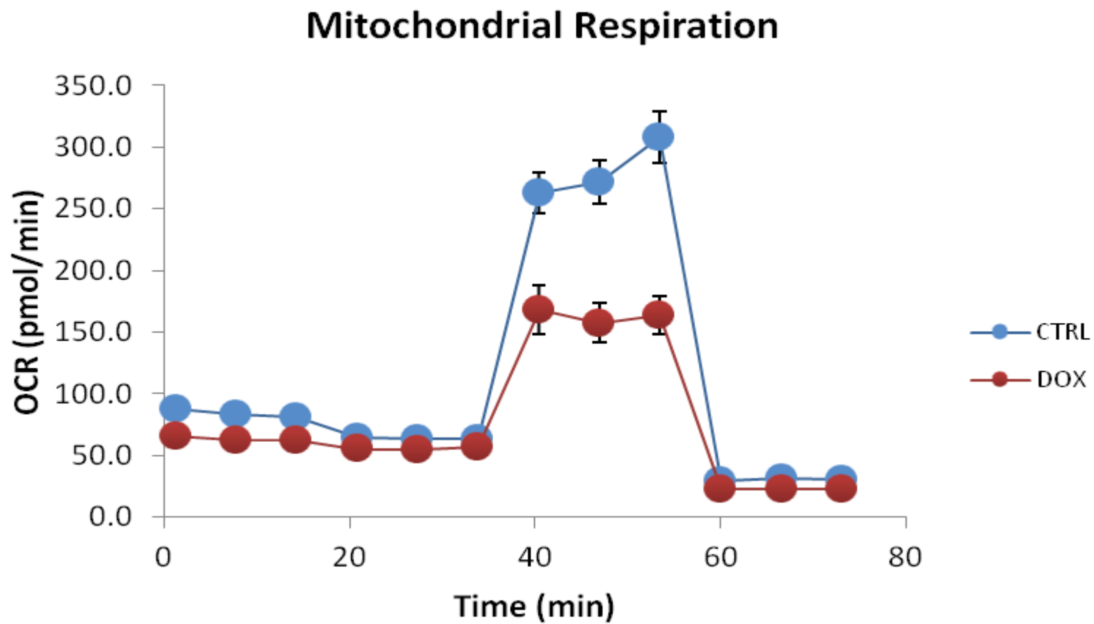
Data are expressed as average ± SEM. Multiple comparisons between groups were determined by one-way ANOVA and Bonferroni *post hoc* test. Unpaired two tailed Student's *t*-test was used to compare mean differences between groups. Differences were considered to be statistically significant to a level of $*P < 0.001$. In all cases, the data were obtained from at least $n = 3–6$ independent myocyte isolations for each condition tested.

IV. Results

1. **Doxorubicin Impaired Mitochondrial Function and Dynamics**

First, we proceeded with assessing the mechanism that underlies the molecular and cellular defects associated with Dox cardiotoxicity. For these studies we treated cardiomyocytes with Dox (1µm) for 24hrs and then analyzed mitochondrial functional end points and cell viability. To begin with we looked at mitochondrial respiration, as shown in Figure 1.1; cells treated with Dox had a significant reduction in maximal respiration and spare respiratory capacity. This impaired respiratory function coincided with a marked increase in fragmented mitochondria (Fig. 1.2) signifying enhanced mitochondrial fission. This was verified through western blot analysis (Fig. 1.2) where phosphorylation of the mitochondrial fission protein DRP1 was significantly increased indicating that DRP1 was activated. Notably, this coincided with a reduction in mitochondrial fusion proteins such as MFN2. In addition, Dox adversely affected the NF-κB signalling pathway. As shown by Western Blot analysis we observed a marked reduction in NF-κB p65 subunit protein expression as well as a reduction in constitutively active subunits of the IKK-NF-κB signaling complex protein IKKβ in cells treated with Dox. (Fig. 1.2B)

A



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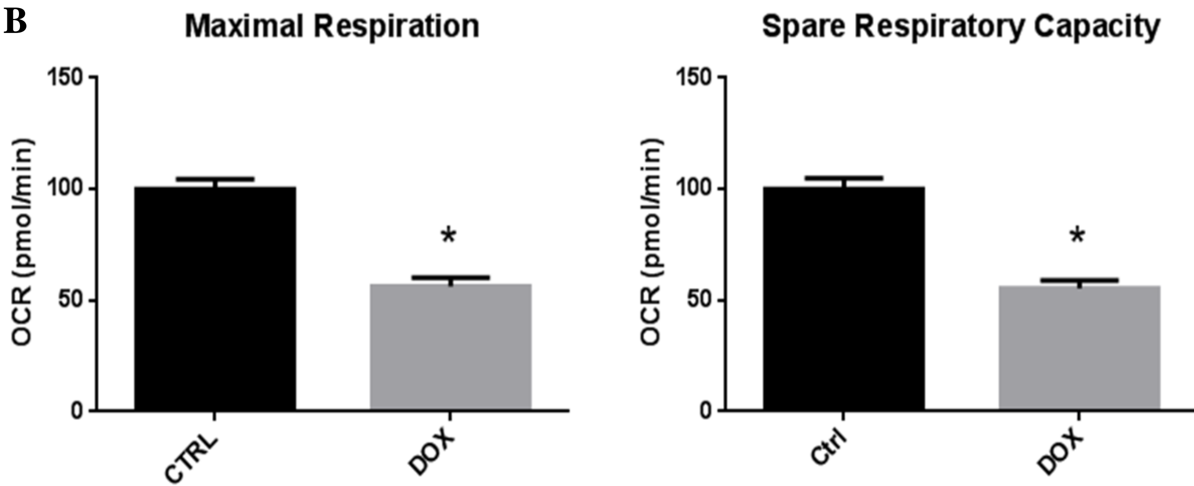

Figure 1.1 Doxorubicin causes impaired mitochondrial respiratory function

Panel A: Mitochondrial Respiration was measured for cardiac myocytes in the presence and absence of Dox after 24hrs using XF96 Seahorse metabolic analyzer **Panel B:** Histogram represents quantitative analysis of data from Panel A, data are expressed as mean±S.E. from at least n=3 independent myocyte isolations. * denotes p<0.0001 compared to control

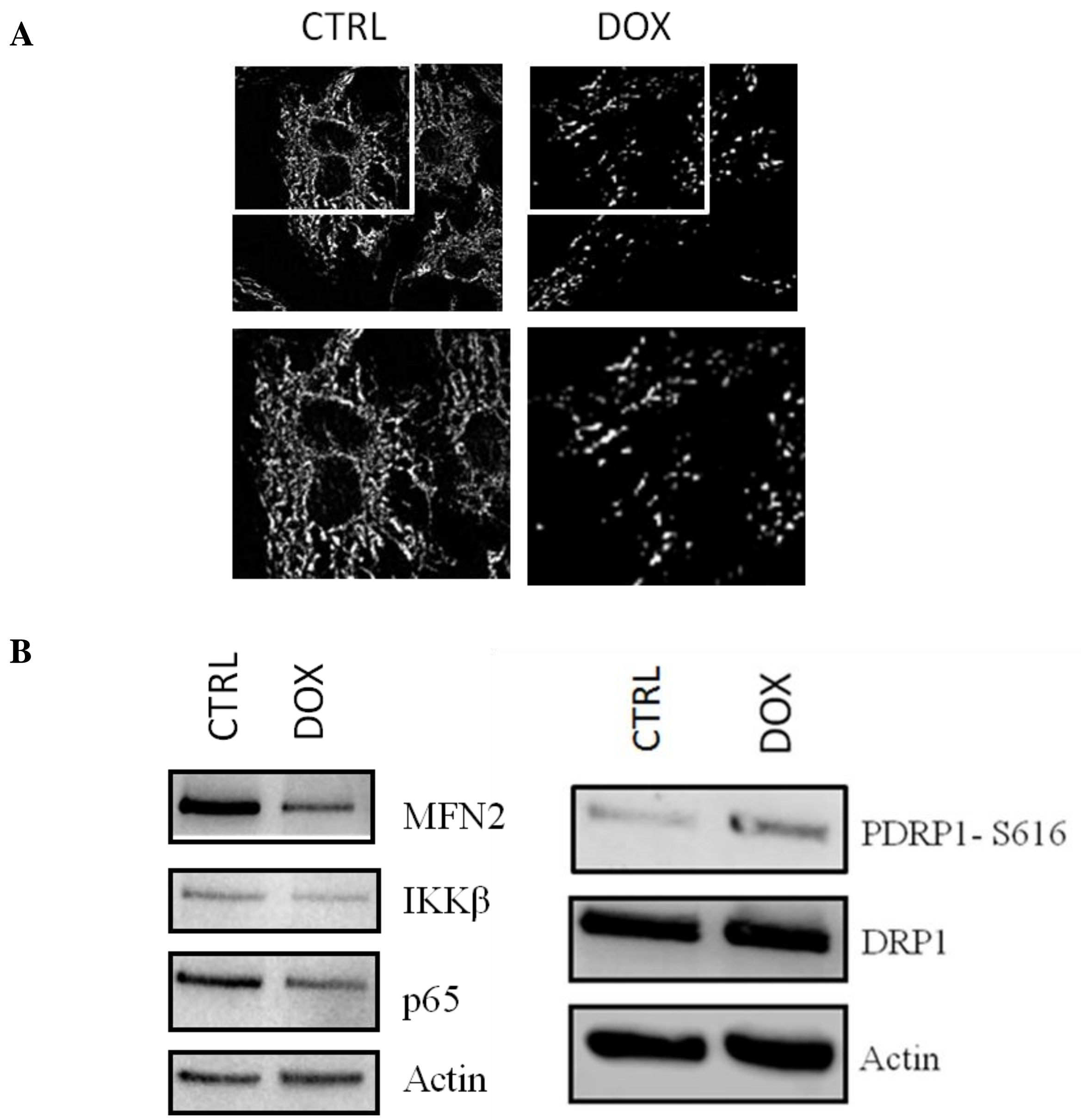

Figure 1.2 Doxorubicin causes impaired Mitochondrial Dynamics and impaired NF-κB signaling

Panel A: Representative images of cardiac myocytes in the presence and absence of Dox analyzed for mitochondrial morphology following immunostaining with Hsp60 antibody. **Panel B:** Western blot analysis of cardiomyocytes in the presence and absence of Dox. The blot was probed with antibodies directed against pDRP1, DRP1, MFN2, IKKβ and p65 subunit of NF-κB. Actin served as a loading control.

2. **Autophagy Mediated Removal of MFN2 Sensitizes Cardiac Myocytes to Doxorubicin**

We previously saw that Dox resulted in down regulation on the outer mitochondrial membrane protein MFN2 which is important for regulating mitochondrial fusion and network assembly. This raised the possibility that lack of MFN2 in cardiac myocytes treated with DOX could underlie the observed deterioration of mitochondrial function. To address this possibility, we observed that when MFN2 was over-expressed in cardiac myocytes treated with Dox it protected against Dox cytotoxicity and rescued cell viability. (Fig. 2.1) To begin to address doxorubicin's effect on MFN2 activity, we performed several experiments to assess the mode by which Dox may be adversely influencing MFN2 protein expression levels and degradation pathway. First, we assessed whether MFN2 mRNA expression levels were altered in cardiomyocytes treated with Dox after 24hrs. As determined by qPCR analysis, we observed that MFN2 mRNA levels were minimally affected by Dox (Fig 2.2A), indicating that the observed decline in MFN2 protein expression levels in cells treated with Dox was not related to altered transcriptional impairment of MFN2 and points to the possibility that DOX influences the protein stability of MFN2. To test this possibility, we next, we tried to decipher if MFN2 was being cleared through proteasomal degradative process. For these studies, we assessed MFN2 protein expression levels in cardiac myocytes treated with Dox in the presence and absence of Lactacystin to inhibit the 26 S proteasome. As shown in Fig 2.2 panel B, western blot analysis of cardiomyocytes treated with Lactacystin demonstrated that under proteasomal inhibition MFN2 protein level was still degraded. This indicates us that MFN2 in not degraded by a proteasomal mediated pathway in cardiac myocytes treated with Dox stress. Given these remarkable findings, we reasoned that an alternative protein degradative pathway may be operating to degrade MFN2. Since autophagy is a cellular mechanism for degradation of cellular proteins under stress conditions, we investigated

whether MFN2 is degraded by an autophagic pathway. To address this possibility we designed short hairpin RNA (shRNA) directed against ATG7 which is critical for autophagy induction and function as a means to inhibit autophagy[136,137] As shown in Fig 2.2 panel C, we observed that when cardiomyocytes were treated with an adenovirus encoding shATG7 to knock-down ATG7 in the presence and absence of Dox, we noted that MFN2 levels are maintained suggesting that MFN2 is degraded in an autophagic dependent manner in cardiac myocytes treated with Dox. Although the possibility existed that MFN2 could be degraded through mitochondrial specific pathway involving autophagy (mitophagy), we assessed the expression levels of other mitochondrial proteins such as voltage dependent anion channel (VDAC) or translocase of outer membrane receptor 20 (TOM 20) both of which are highly conserved in the mitochondria[138,139]. We also assessed a protein of Complex I NADH: Ubiquinone Oxidoreductase Subunit A9 (NDUFA9). We observed no apparent change in the expression levels of these proteins (Fig 2.2D), excluding the possibility that the loss of MFN2 protein expression in cardiac myocytes treated with Dox is related to global mitochondrial clearance through mitophagy.

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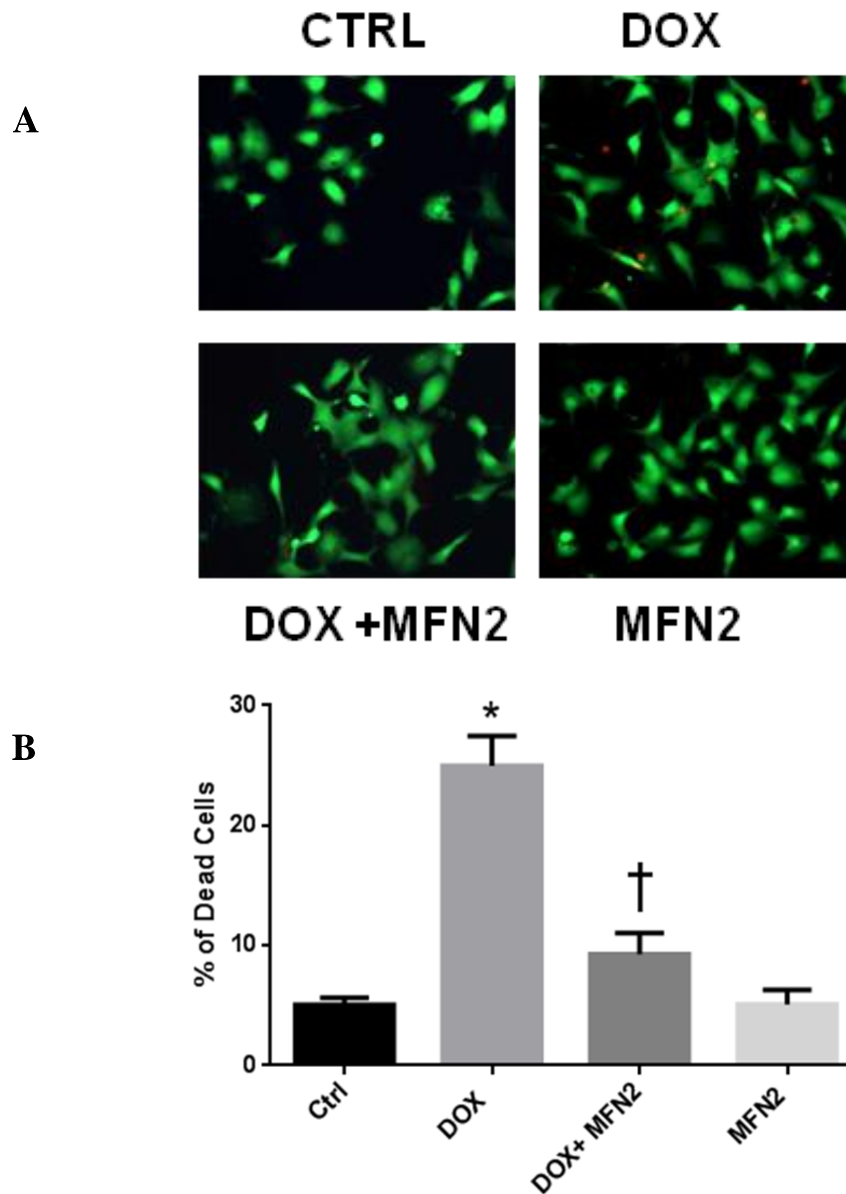

Figure 2.1 MFN2 is protective against Dox induced cell death

Panel A: Representative epifluorescent images of cardiomyocytes stained with vital dyes calcein-AM and ethidium homodimer-1 to detect the number of live cells (green) and dead cells (red). **Panel B:** Histogram represents quantitative data shown, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes $p<0.0001$ compared to control. †denotes $p<0.0001$ compared to Dox

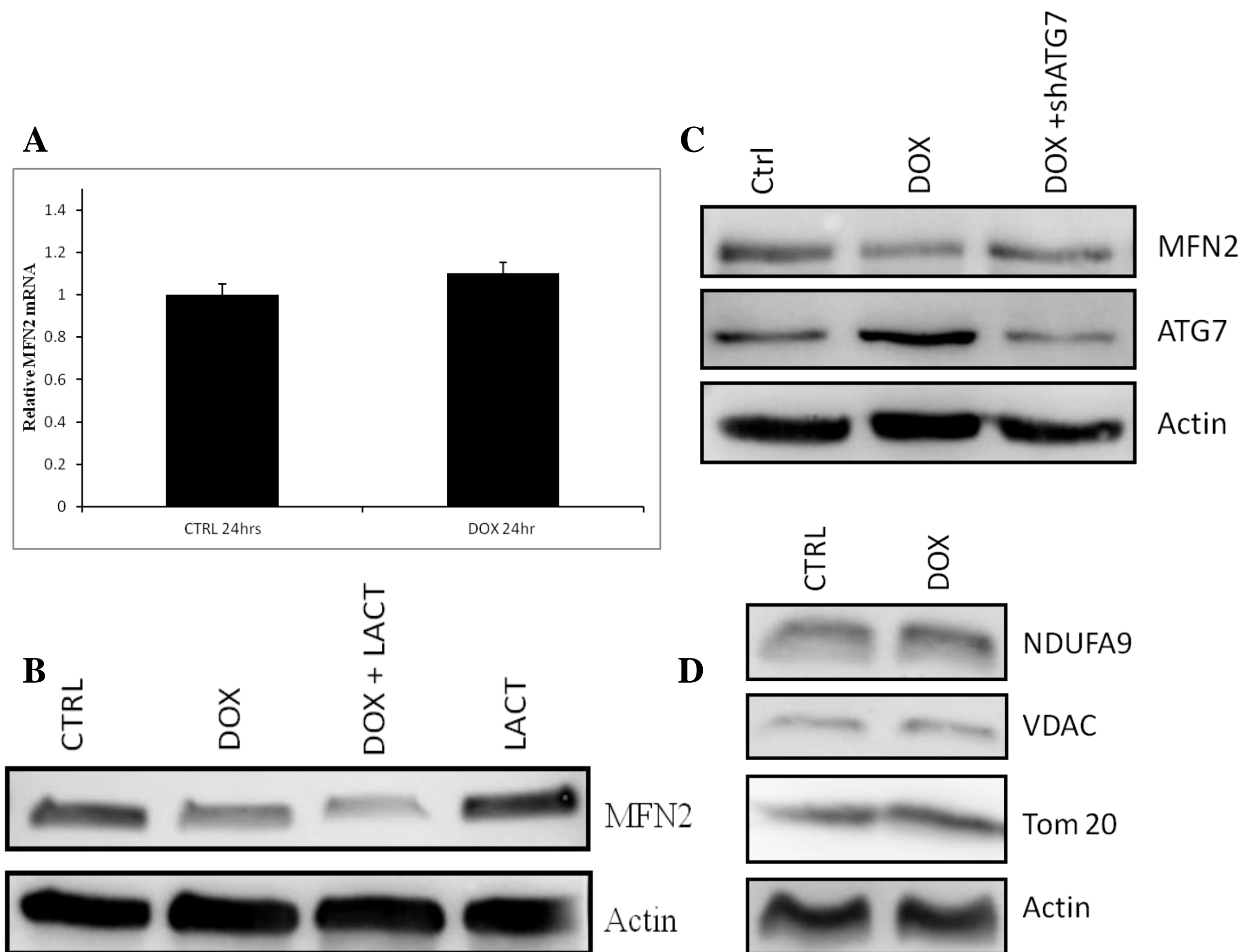

Figure 2.2 Autophagy Mediated Removal of MFN2 Sensitizes Cardiac Myocytes to Doxorubicin

Panel A: MFN2 mRNA expression of cardiac cells in the presence and absence of Dox. **Panel B:** Western blot analysis of cardiac cell lysate for MFN2 and alpha Actin as loading control in the presence of or absence of Lactacystin. **Panel C:** Western blot analysis of cardiac cells treated with shATG7 adenovirus in the presence and absence of Dox. The blot was probed for antibodies against MFN2 and ATG7, alpha Actin served as loading control. **Panel D:** Western blot analysis of cardiac cells in the presence and absence of Dox. Antibodies targeted against mitochondrial proteins VDAC and TOM 20 were probed on the membrane, alpha Actin served as loading control

3. **IKKβ Interacts with and Prevents MFN2 Degradation in Cardiac Myocytes Treated with Doxorubicin.**

Since previous work in our lab has shown that IKKβ is cytoprotective and protect cardiac myocytes during hypoxic stress, we ascertained whether IKKβ would protect cardiac myocytes were treated with Dox. As shown in Fig 3.1 over-expression of IKKβ wild type in IKKβ (IKKβ wt), preserved MFN2 expression in cardiac myocytes treated with Dox. Interestingly when cardiac myocytes expressed a kinase deficient mutant of IKKβ ($IKK\beta_{k/m}$) and treated with Dox, MFN2 levels were not maintained and MFN2 levels declined as observed in virally infected control cells. Based on these findings we decided to perform a co-immunoprecipitation (co-IP) assay to test if IKKβ interacted with MFN2 to post-translationally modify its stability. For these studies, cell lysate from vehicle-treated cells or cells over expressing IKKβ and/or IKKβ and MFN2 was subjected to IP. As shown in Figure 3.1, a notable interaction between the IKKβ and MFN2 was found in all three conditions. Since we observed a strong interaction between the IKKβ and MFN2 we wanted to further investigate whether IKKβ post translationally modifies MFN2. To address this goal, we performed mass spectroscopy analysis on HEK293s over expressing IKKβ or kinase inactive IKKβ mutant $IKK\beta_{k/m}$. Mass spec analysis revealed that IKKβ wt but not $IKK\beta_{k/m}$ phosphorylated serine 53 on the MFN2 protein (Fig 3.2), verifying that IKKβ post-translationally modifies MFN2 activity which influences MFN2 protein stability and autophagic degradation. To assess whether preserving MFN2 activity would influence mitochondrial function we assessed the impact of IKKβ regulated MFN2 activity on mitochondrial morphology and mitochondrial respiration. As shown in Figure 3.3 and3.4, IKKβ wt rescued mitochondrial morphological and respiratory defects induced by Dox. Taken together

our data suggest IKKβ rescued mitochondrial perturbations induced by Dox through a mechanism related directly to MFN2 activity and dependent upon the kinase activity of IKKβ.

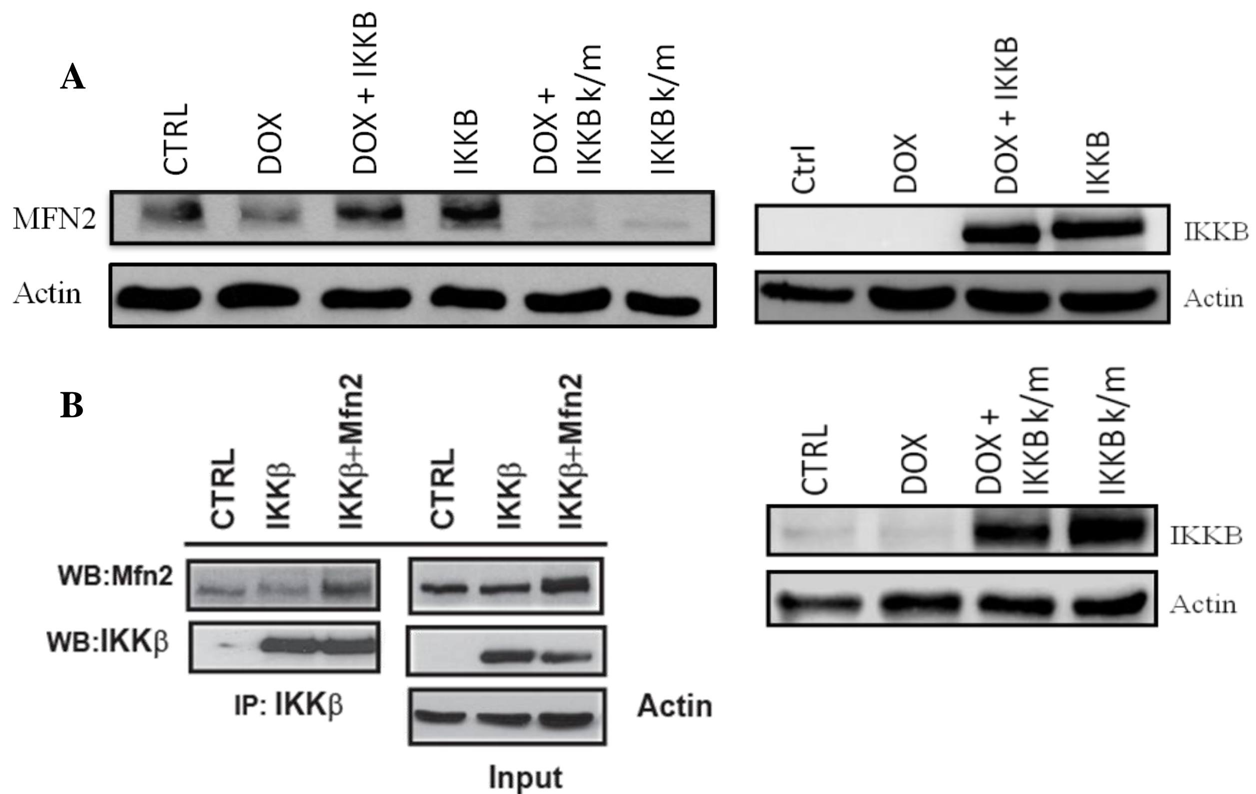

Figure 3.1 IKKβ interacts and prevents MFN2 degradation under Dox

Panel A: Western blot analysis of cardiac cell lysate treated with and without DOX in the presence and absence of IKKβ wt and $IKK\beta_{k/m.}$ The filter was probed with antibodies directed against MFN2, IKKβ and alpha Actin. **Panel B:** Protein lysate derived from HEK293 cells over expressing IKKβ and MFN2 were immunoprecipitated with an antibody directed against IKKβ and probed with antibody directed against MFN2. Western blot of cell lysate used for Immunoprecipitation were analyzed in parallel for expression of IKKβ, MFN2, and Actin proteins

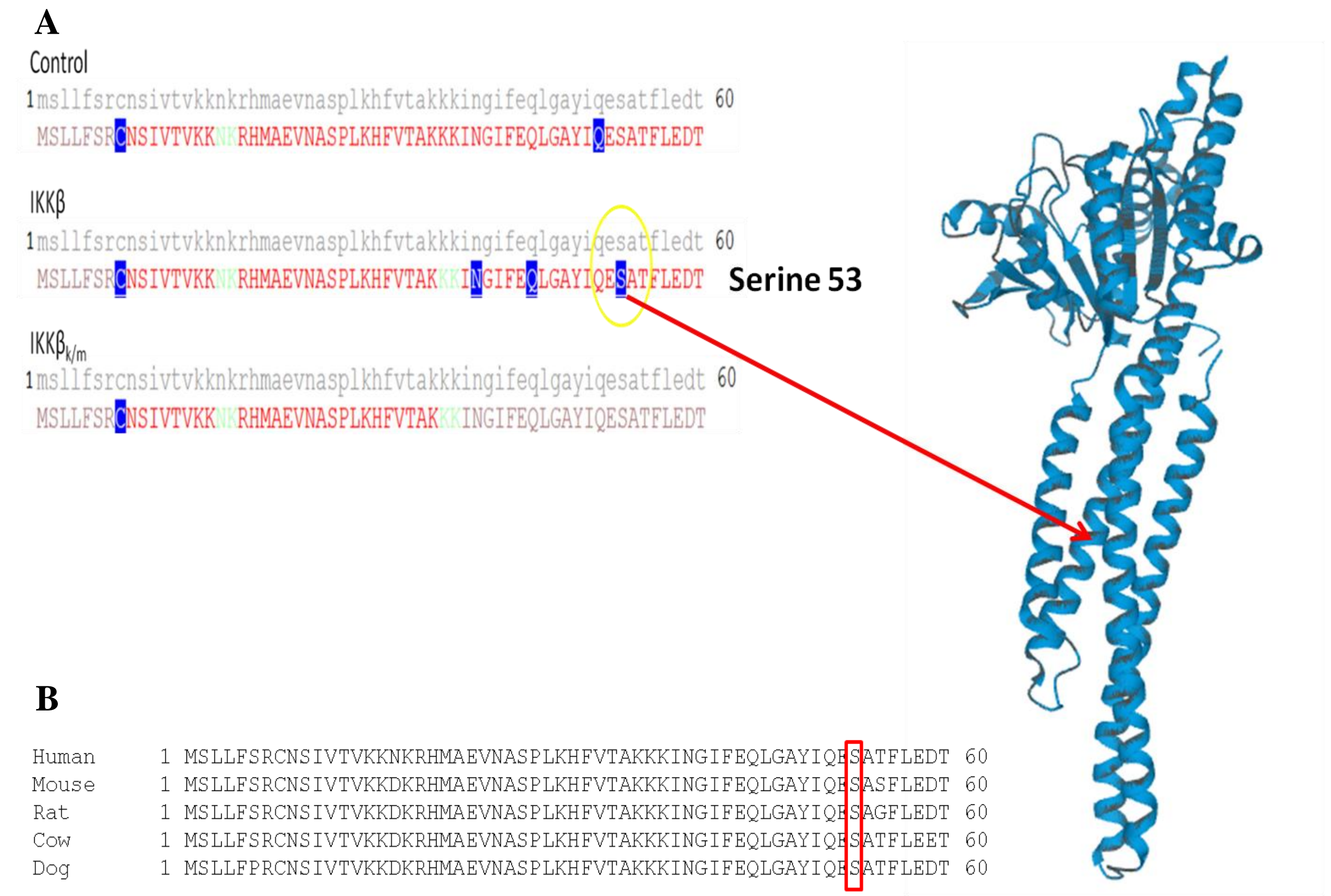

Figure 3.2 IKKβ interacts and phosphorylates MFN2.

Panel A: Mass spectroscopy analysis showing results for post translational modifications on the MFN2 protein. Cell lysate from HEK293 cells enriched with MFN2 were analyzed in the presence and absence of IKKβ wt and IKK$\beta_{k/m}$. Ribbon structure of MFN2 as previously reported by (Li, Y.J., et al. 2019), Protein Data Bank-PDB ID: 6JFM[140] **Panel B:** Alignment across multiple species showing that serine 53 is conserved.

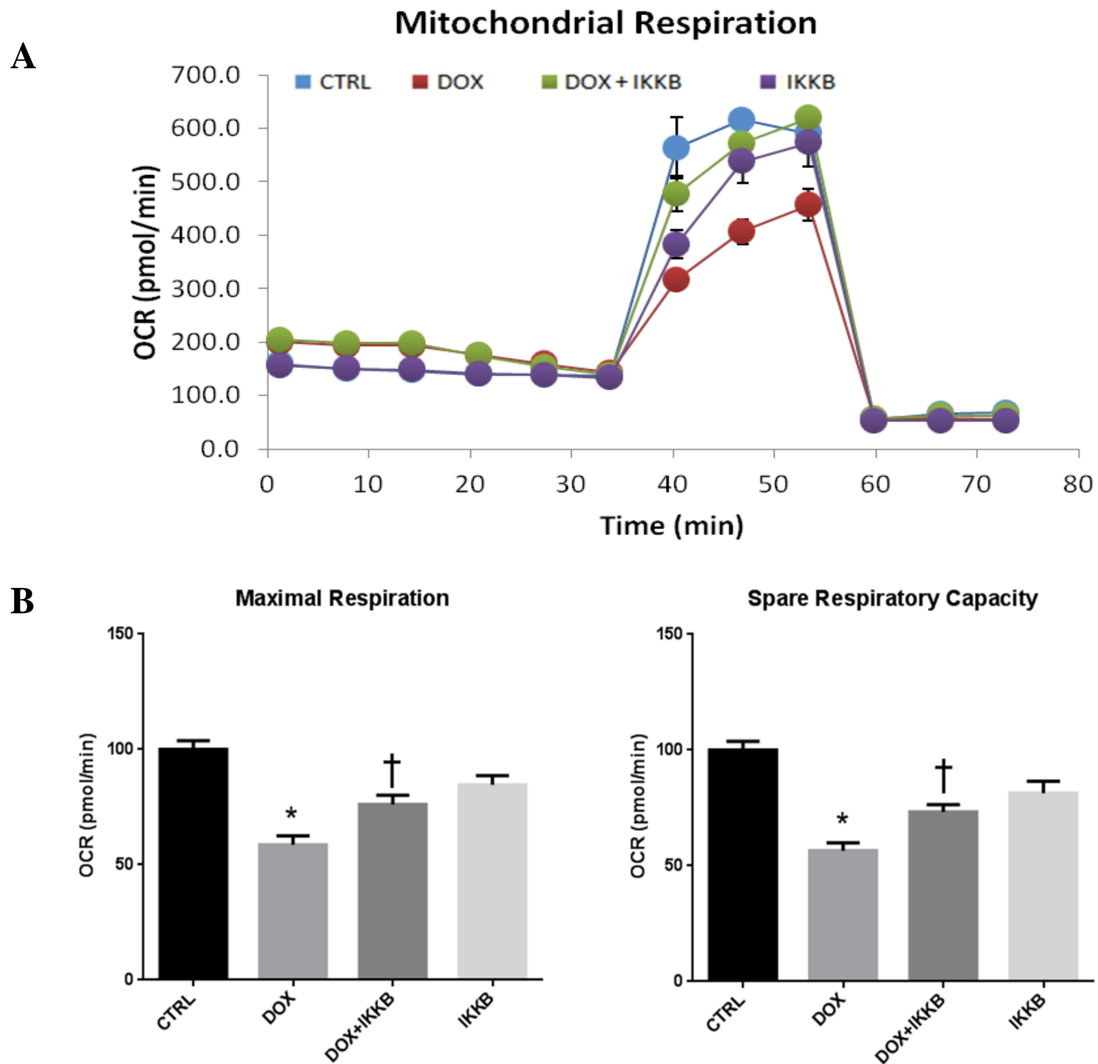

Figure 3.3 IKKβ rescues Dox induced impaired mitochondrial respiration

. **Panel A:** Mitochondrial respiration was measured for cardiac myocytes treated with IKKβ wt in the presence and absence Dox using XF96 analyzer. **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes $p<0.0001$ compared to control. †denotes $p<0.0001$ compared to Dox. ‡ denotes $p<0.0001$ compared to Dox+ IKKβ

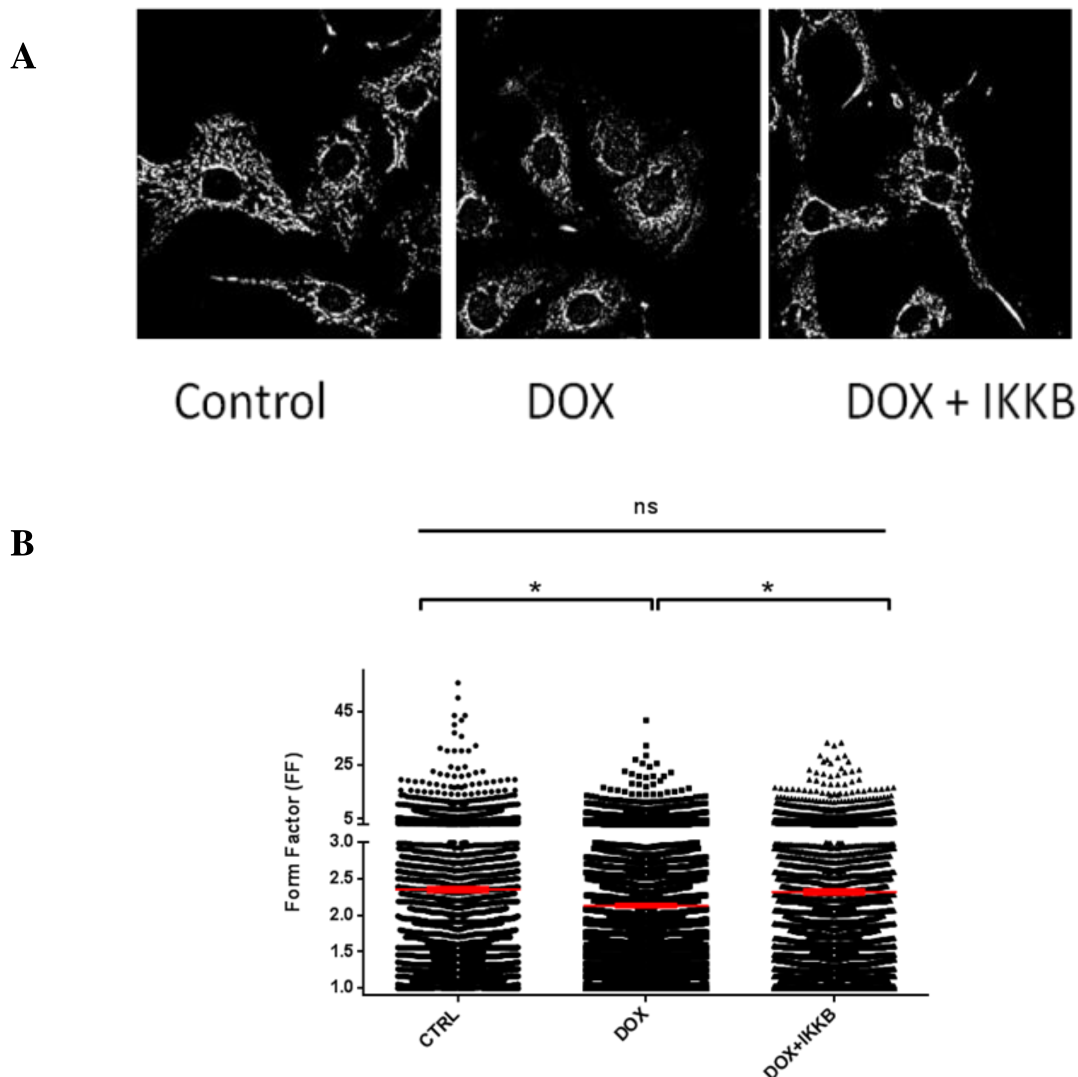

Figure 3.4 IKKβ prevents excessive mitochondrial fragmentation due to Dox

Panel A: Representative images of cardiac myocytes analyzed for mitochondrial morphology following immunostaining with Hsp60 antibody. **Panel B**: Histogram represents quantitative data for mitochondrial morphology. The graph shows mitochondrial form factor, (FF), an index of mitochondrial fission for cells analyzed per condition, low FF values are indicative of mitochondrial fission. Histogram represents quantitative data shown in, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes $p<0.0001$ compared to control.

4. **IKKβ Maintained Mitochondrial Function and Prevented Cell Death**

Given that IKKβ can rescue mitochondrial fusion and respiration, we next assessed whether IKKβ could rescue functional aspects of the mitochondria. As shown in Fig.4.1-4.3 under conditions where cardiac myocytes were treated with Dox, IKKβ had a significant positive effect on mitochondrial function. We observed that Dox induced increase of ROS production, mPTP opening, and loss of mitochondrial membrane potential (ΔΨm) were rescued by IKKβ wt. In contrast,-the kinase deficient mutant of IKKβ ($IKKβ_{k/m}$) had no effect on Dox induced mitochondrial defects. These findings verify that the IKKβ kinase activity is necessary and sufficient to restore mitochondrial defects induced by Dox. Since IKKβ was able to suppress mitochondrial damaged induced by Dox, we further assessed whether over-expression of IKKβ would result in improved cell viability and reduce the incidence of cell death induced by DOX. As shown in Fig.4.4 cell viability assay in cardiac myocytes expressing IKKβ wt or $IKKβ_{k/m}$ in the presence or absence of Dox demonstrated that IKKβ wt but not the kinase inactive mutant was able to suppress cell death induced by Dox. Collectively, the data substantiate a cytoprotective role for IKKβ wt against Dox induced cardiotoxicity.

A

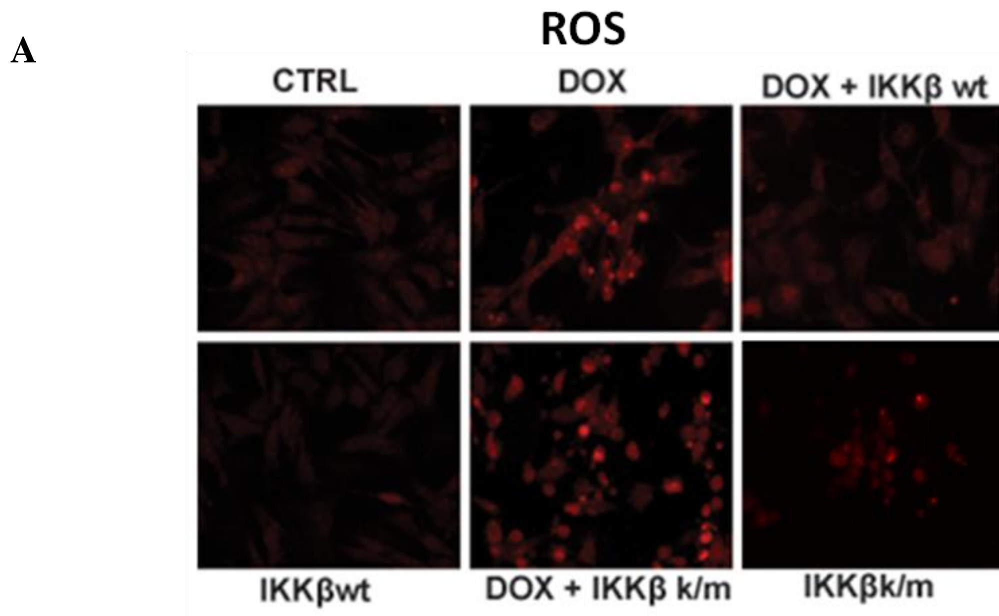

Figure 4.1 IKKβ prevents increased ROS production due to Dox

Panel A: Epifluorescence microscopy for ROS production of ventricular myocytes expressing IKKβ wt or $IKKβ_{k/m}$ in the presence or absence of Dox

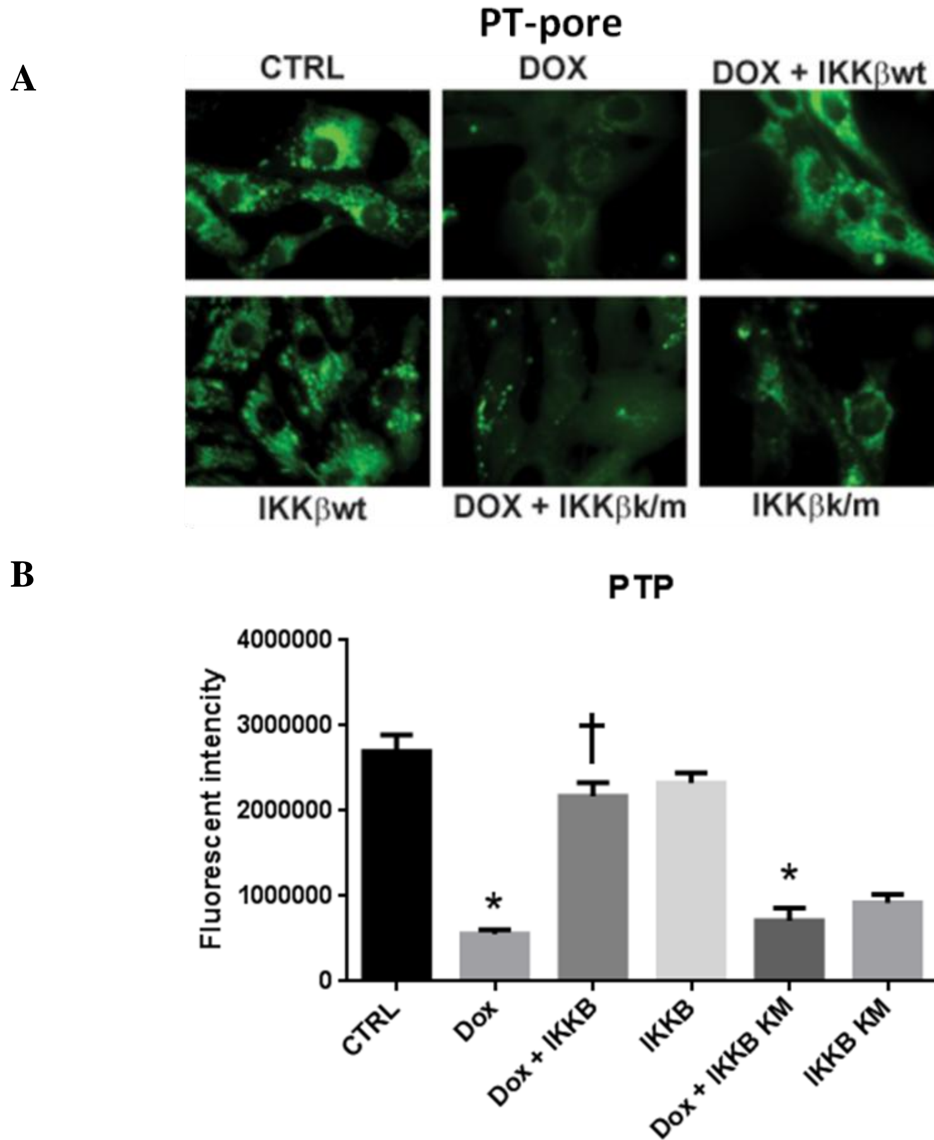

Figure 4.2 IKKβ maintains mitochondrial PT-pore function

Panel A: mPTP opening was assessed in cells expressing IKKβ wt or $IKK\beta_{k/m}$ in the presence or absence of Dox. **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes $p<0.0001$ compared to control. †denotes $p<0.0001$ compared to Dox

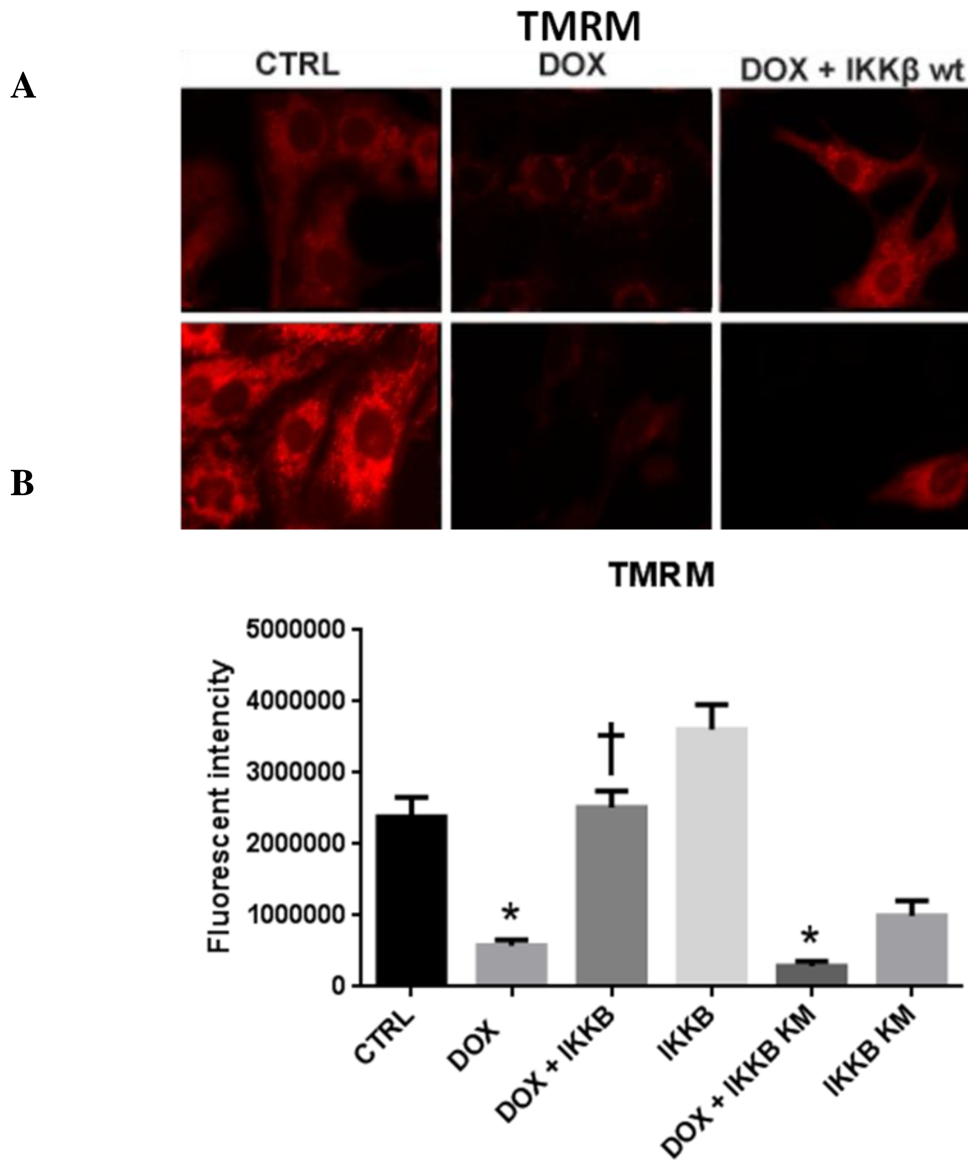

Figure 4.3 IKKβ restores loss of Mitochondrial Membrane Potential (ΔΨm)

Panel A: Epifluorescence microscopy for mitochondrial membrane potential (ΔΨm) was assessed in cells expressing IKKβ wt or IKK$\beta_{k/m}$ in the presence or absence of Dox. **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes $p<0.0001$ compared to control. †denotes $p<0.0001$ compared to Dox

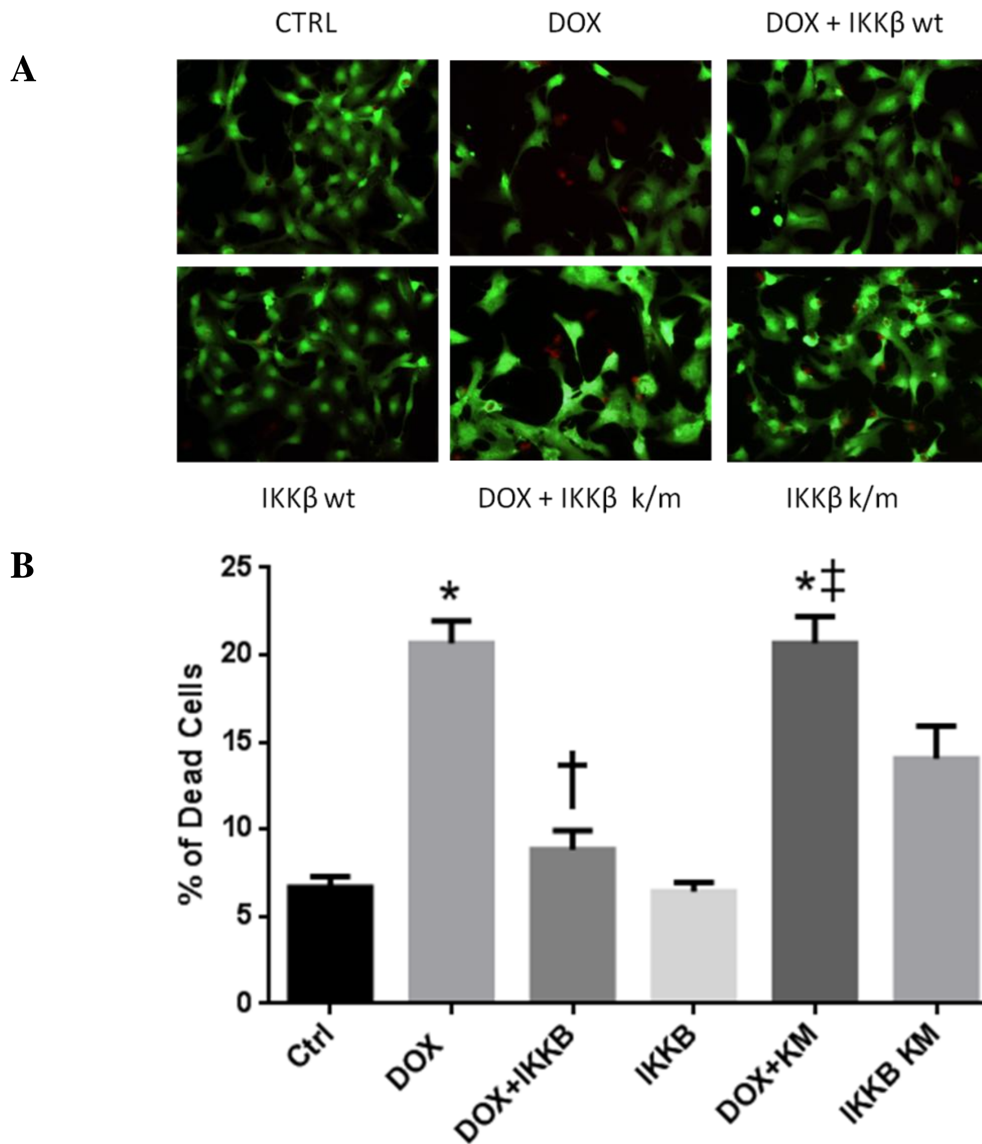

Figure 4.4 IKKβ prevents Dox induced cardiac cell death

Panel A: Representative epifluorescent images of cardiomyocytes treated with and without DOX in the presence and absence of IKKβ wt and $IKK\beta_{k/m.}$ Cells were stained with vital dyes calcein-AM and ethidium homodimer-1 to detect the number of live cells (green) and dead cells (red). **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes p<0.0001 compared to control. †denotes p<0.0001 compared to Dox. ‡ denotes p<0.0001 compared to Dox+ IKKβ

5. **IKKβ protective effects of mitochondrial function is impaired when MFN2 is inactive**

Surprisingly IKKβ protective effects of mitochondrial respiration were diminished when a GTPase defective form of MFN2 (MFN2 aa) was used. (Fig. 5.1) This shows that when MFN2 is impaired IKKβ is unable to prevent Dox induced mitochondrial perturbations. Cell viability was also assessed for this and showed that when MFN2 was impaired IKKβ was unable to rescue cardiac cells when they were also treated with Dox. (Fig 5.2) This again links IKKβ cell survival pathway to its interaction and association with MFN2.

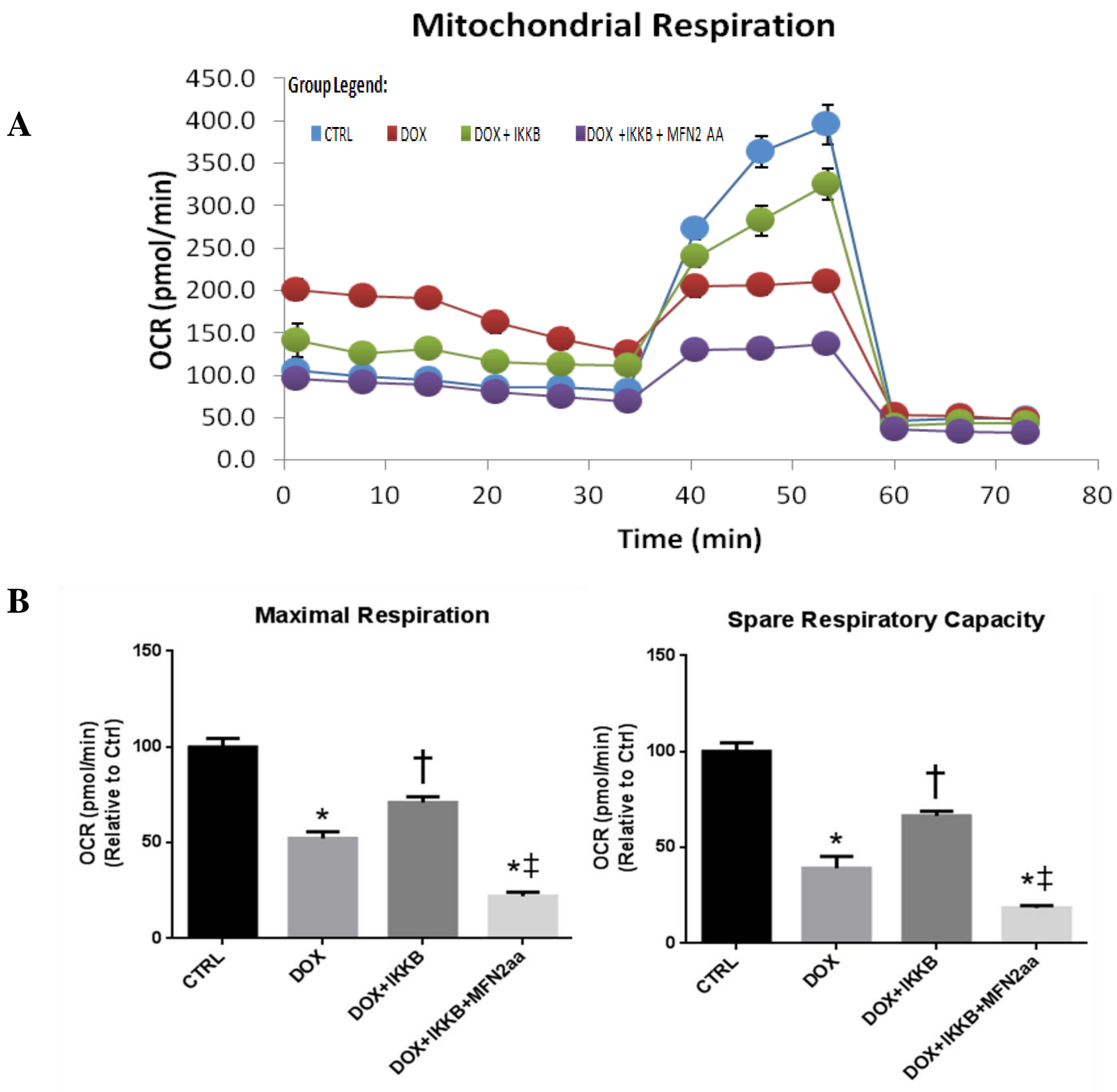

Figure 5.1 IKKβ protective effects on mitochondrial function is impaired when MFN2 is inactive

Panel A: Mitochondrial respiration was measured for myocytes expressing IKKβ wt and MFN2 aa in the presence of Dox, using the XF96 Seahorse metabolic analyzer. **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes p<0.0001 compared to control. †denotes p<0.0001 compared to Dox. ‡ denotes p<0.0001 compared to Dox+ IKKβ

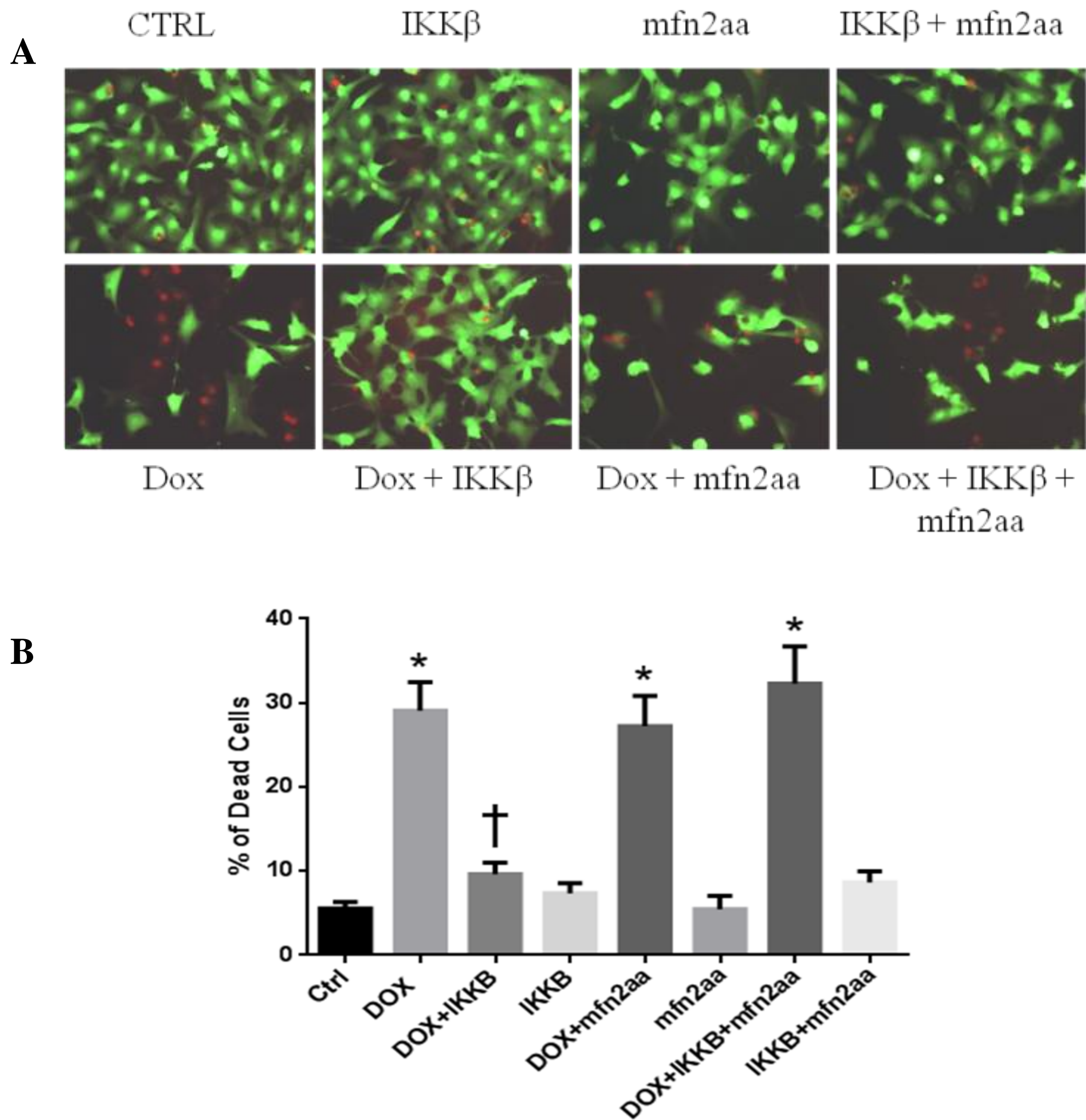

Figure 5.2 IKKβ protective effects on cell viability is impaired when MFN2 is inactive

Panel A: Representative epifluorescent images of cardiomyocytes treated with and without DOX in the presence and absence of IKKβ wt and MFN2aa. Cells were stained with vital dyes detect the number of live cells (green) and dead cells (red). **Panel B:** Histogram represents quantitative data shown in Panel A, data are expressed as mean±S.E.M from at least n=3 independent myocyte isolations. * denotes p<0.0001 compared to control. †denotes p<0.0001 compared to Dox

V. Discussion

Doxorubicin is a highly effective chemotherapy agent used to treat a variety of human cancers. However, a major side effect of doxorubicin treatment that has been known and well established is its cardiotoxicity. Indeed, a major limitation associated with doxorubicin and related Anthracyclines is cardiac dysfunction commonly observed in many patients treated with this drug. In fact, many of the consequences of doxorubicin cardiotoxicity are the impaired cardiac function[141] and heart failure[29] that ensues following doxorubicin treatment. However, the underlying mechanisms remain poorly understood. Given that the heart relies heavily on a constant supply of ATP to maintain normal cardiac function, it is not surprising that defects in energy metabolism may be a contributing underlying cause[142]. In this regard, the mitochondrion is a major source of ATP production in the heart through oxidative metabolism and therefore mitochondrial fitness is critical for the cell to maintain a healthy pool of mitochondria to support vital cellular processes essential for cell survival. Defects in mitochondrial fitness can lead to cardiac dysfunction and heart failure[143,144]. Since the heart in contrast to other organs of the body are heavily enriched in mitochondria. For this reason, we focused our attention on the mitochondrial function as an underlying cause of doxorubicin cardiomyopathy. Mitochondrial fitness refers to a critical process that includes mitochondrial dynamics, bioenergetics and function. Mitochondrial function is impaired through the uncoupling of electron transport proteins and mitochondrial respiration can trigger excessive ROS production which has been suggested to contribute to Doxorubicin cardiomyopathy[6]. Mitochondrial metabolism is regulated by continual fusion and fission of mitochondria which is governed by a number of proteins on the outer and inner mitochondrial proteins[145]. Mitochondrial fusion is mediated by the larger dynamin GTPase proteins MFN1 and MFN2 on the outer mitochondrial membrane and OPA1 on

the inner mitochondrial membrane. In contrast Dynamin related protein DRP1 promotes mitochondrial fission. The coordinated action of MFN1/2 and DRP1promotes mitochondrial fusion or fission and is responsible for regulating mitochondrial morphology and networks. Interestingly, in addition to regulating mitochondrial fusion, MFN2 can also regulate other cellular processes such as ER tethering[104] and stress induced cell survival and death[75,146]. Loss of MFN2 but not MFN1 has been shown to lead to increased mitochondrial fission and ROS production in cardiac cells[119]. Hence, loss of mitochondrial fusion events and increased fission resulting in disruption of mitochondrial networks from loss of MFN2 activity may be a critical underlying defect that leads to mitochondrial dysfunction and cell death associated with doxorubicin cardiotoxicity. The link between mitochondrial dysfunction from loss of MFN2 activity is poorly defined. Interestingly, previous studies from our lab identified a novel survival role the NF-κB signaling pathway in cardiac myocytes. Indeed, we previously reported that NF-κB activation promotes a survival signaling pathway in cardiac myocytes. Loss of NF-κB signaling has been associated with impaired viability and increased cell death of cardiac myocytes[135]. NF-κB is regulated in mammalian cells including cardiac myocytes by the phosphorylation dependent degradation of the NF-κB inhibitor protein IκBα. Phosphorylation of IκBα at critical serine residues 32 and 36 by the IKK signaling complex promote the proteasomal degradation of IκBα[147]. The loss of IκBα permits the cytoplasmic translocation of NF-κB to the nucleus where it affects gene transcription. Therefore, the IKK signaling complex which is comprised of IKKα, IKKβ and IKKγ (NEMO) represents an important mechanism for NF-κB activation and promoting cell survival. This view is supported by the fact that IKKβ was shown to activate NF-κB in cardiac myocytes and importantly suppress mitochondrial perturbations including loss of mitochondrial membrane potential, permeability transition pore

opening and cell death of cardiac myocytes during hypoxia[14]. The fact that IKKβ alone was sufficient to suppress mitochondrial defects in cardiac myocytes subjected to hypoxia, raised the interesting possibility that IKKβ may promote cell survival by preserving mitochondrial fitness. Based on these observations we tested whether IKKβ which has been shown to promote cell survival of cardiac myocytes, could suppress mitochondrial injury and cell death observed in cardiac myocytes treated with doxorubicin.

In this study we provided new evidence for the cytoprotective role of IKKβ, a key protein in the NF-κB signaling pathway for suppressing mitochondrial injury in cardiac myocytes treated with doxorubicin. Herein, we show that IKKβ elicits its protective effects against Doxorubicin by maintaining mitochondrial integrity. We further show that our study mechanistically demonstrates that IKKβ regulates mitochondrial fitness by controlling the mitochondrial fusion protein MFN2. Importantly, we show that IKKβ directly forms a novel protein-protein with the outer mitochondrial membrane protein MFN2 which is critical for IKKβ mediated cardioprotection against doxorubicin cardiotoxicity. Another important feature of our study was the finding that the kinase activity of IKKβ was shown to be important for regulating MFN2 protein stability and therefore maintaining the mitochondrial dynamics in Doxorubicin treated cardiomyocytes. This view is supported by the finding that the wild type IKKβ but not the kinase inactive $IKK_{k/m}$ suppressed mitochondrial injury and cell death induced by doxorubicin. Notably, mass spectroscopy analysis revealed that that the wild type IKKβ phosphorylated MFN2 which was important for regulating mitochondrial fusion and rescuing mitochondrial electron transport and respiration. This coincided with improved mitochondrial morphology and cell viability of cardiac myocytes treated with doxorubicin.

Previous reports from our lab have shown that hypoxic stress was primarily caused by mitochondrial defects which ultimately led to cardiac cell death[14]. Our lab also demonstrated a cardioprotective role for IKKβ-NF-κB signaling pathway in cardiac myocytes against hypoxic stress through preserving mitochondrial integrity, more specifically that the pathway may act on and prevent mPTP opening [14]. Notably, we have observed that IKKβ-NF-κB signaling is disrupted in Dox treated cardiac myocytes. From the current study we see that cardiomyocytes treated with Dox showed reduced metabolic activity and several indications of impaired mitochondrial dynamics resulting in increased mitochondrial fission and impaired respiration. Based on these interesting observations we tested whether IKKβ-NF-κB signaling pathway could regulate Doxorubicin - induced cardiac cell death by maintaining mitochondrial dynamic homeostasis.

Strikingly overexpression of IKKβ wt was able to rescue and more importantly maintain mitochondrial network, membrane potential ($\Delta\Psi m$), mPTP opening and overall reduce ROS production in Doxorubicin treated cardiac cells. Interestingly the kinase mutant $IKK\beta_{k/m}$ failed to rescue this mitochondrial network and functional deficits or cell death leading us to believe that the kinase activity was important for maintaining mitochondrial integrity. Perhaps most compelling was our finding that MFN2 expression which was decreased by Doxorubicin could be rescued by IKKβ but not $IKK\beta_{k/m}$.

Another interesting aspect of our research was that Doxorubicin induced MFN2 degradation through an autophagic process. MFN2 degradation has mostly been attributed to proteasomal degradation[146]. We specifically show that loss of MFN2 protein was suppressed by autophagic inhibition by Atg7 knock down (shATG7) but not by proteasomal inhibition with Lactacystin. The fact that MFN2 expression in cardiac myocytes treated with Doxorubicin was maintained in

the presence of IKKβ raises the possibility that IKKβ might prevent autophagic removal of MFN2 which would provide new insight into MFN2 regulation.

Interestingly the cardioprotective effects of IKKβ was lost in cells expressing a GTPase mutant of MFN2 deficient for mitochondrial tethering which resulted in a decrease in mitochondrial respiration and increased cell death in the presence of with Dox. These novel and important findings highlight the importance of MFN2 in IKKβ mediated cardioprotection and highlight a novel interplay between IKKβ and MFN2 for regulating cell viability cardiac myocytes treated with Dox.

Taken together our study shows for the first time a protective role for IKKβ in maintaining mitochondrial dynamics of cardiac cells treated with Dox. Additionally, we identified a novel interaction between IKKβ and MFN2 that prevents Dox induced autophagic degradation of MFN2 leading to subsequent mitochondrial dysfunction and cell death. Therefore, interventions that target IKKβ-MFN2 signalling in cardiomyocytes may prove beneficial in preventing cardiac issues developed in cancer patients undergoing Dox chemotherapy.

VI. References

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