DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

Programa de Doctorado de Biociencias y Ciencias Agroalimentarias

Alterations in growth and cell death by genetic deletion of the antioxidant response factor Nrf2 and its target, the quinone reductase NQO1

Alteraciones del crecimiento y la muerte celular producidas por la deleción genética del factor de respuesta antioxidante Nrf2 y de su diana, la quinona reductasa NQO1

Julia Ariza Gómez

Córdoba, 2015

TITULO: *Alterations in growth and cell death by genetic deletion of the antioxidant response factor Nrf2 and its target, the quinone reductase NQ01*

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Alterations in growth and cell death by genetic deletion of the antioxidant response factor Nrf2 and its target, the quinone reductase NQO1

Memoria de Tesis Doctoral presentada por **Julia Ariza Gómez**, Licenciada en Biología, para optar al grado de **Doctora en Ciencias**.

El Director,

Dr. José Manuel Villalba Montoro

Catedrático de Biología Celular
Universidad de Córdoba

En Córdoba, a 28 de mayo de 2015

UNIVERSIDAD DE CÓRDOBA

D. José Manuel Villalba Montoro, Doctor en Ciencias y Catedrático de Universidad del Área de Biología Celular del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba,

INFORMA

Que Julia Ariza Gómez, Licenciada en Biología, ha realizado bajo su dirección el trabajo titulado **"Alterations in growth and cell death by genetic deletion of the antioxidant response factor Nrf2 and its target, the quinone reductase NQO1"**, que a su juicio reúne los méritos suficientes para optar al Grado de Doctora en Ciencias.

Y para que conste, firma el presente INFORME en Córdoba, a 28 de mayo de 2015.

Fdo.: José Manuel Villalba Montoro

TÍTULO DE LA TESIS:

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DOCTORANDA: Julia Ariza Gómez

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

Durante el desarrollo de la presente Tesis Doctoral, llevada a cabo entre los años 2011 y 2015, la doctoranda Julia Ariza Gómez ha superado con creces los objetivos, tanto formativos como de investigación, planteados al comienzo de la misma. La expresión génica dependiente del factor de transcripción Nrf2 constituye uno de los mecanismos esenciales que permiten a las células responder ante diversas situaciones de estrés, particularmente en condiciones de estrés oxidativo. Entre las distintas dianas de Nrf2 encontramos la quinona reductasa NQO1, la cual es capaz de reducir un gran número de sustratos quinónicos usando NAD(P)H. Además de en la defensa frente al estrés, Nrf2 y NQO1 también participan en el control del balance redox celular, por lo que también están implicados en la regulación de sistemas de señalización relacionados con en el crecimiento celular. El trabajo desarrollado por la doctoranda ha permitido poner de manifiesto que la deleción de Nrf2 y de NQO1 altera profundamente el patrón de crecimiento de las células. Aunque en ambos casos existe un incremento de la apoptosis celular, la capacidad de crecimiento resulta disminuida en las células carentes de Nrf2, pero aumentada en las carentes de NQO1. Además, las células carentes de NQO1 mostraron una mayor capacidad migratoria y formaron tumores tras su inyección en ratones inmunodeficientes, lo cual puede explicar la mayor susceptibilidad a la tumorigénesis relacionada con la ausencia de esta reductasa. Por otra parte, la doctoranda ha demostrado que la quinasa p38 regula la expresión de la proteína antiapoptótica Bcl-2 a través de un mecanismo dependiente de Nrf2. Los resultados obtenidos en la Tesis han sido presentados en algunos de los congresos más prestigiosos relacionados con el tema de estudio, tanto a nivel internacional (*European Congress of Biogerontology*, *Oxygen Club of California World Congress*, *Society for Free Radical Research International*) como nacional (*Sociedad Española de Bioquímica y Biología Molecular*, *Sociedad Española de Biología Celular*). Por otra parte, la Tesis ha permitido reunir una cantidad suficiente de información que permitirá publicar los resultados en revistas de alto impacto. Estos artículos se encuentran actualmente en preparación. El trabajo se ha desarrollado en el ámbito de un Proyecto de Excelencia financiado por la Junta de Andalucía. Su formación se ha completado con la realización de colaboraciones de investigación y docentes, y la realización de tres estancias cortas, las dos primeras en el Translational Gerontology Branch (NIA, NIH, Baltimore, USA) y la última en Gladstone Institutes (San Francisco, USA). Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 28 de Mayo de 2015

Fdo.: José Manuel Villalba Montoro

A mis padres, Agustín y Pilar.

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Y llegó el momento de escribir los agradecimientos. Eso quiere decir que ya has escrito todo lo demás… Hace ya más de 10 años que llegué al departamento de Biología Celular y en todo este tiempo, la tercera planta del C-6 se ha convertido en mi casa. No quiero caer en lugares comunes, pero resumir lo que siento en estos momentos me parece tan complicado como los mecanismos de la señalización apoptótica que he tenido que discutir unos días antes.

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Abbreviations

ADP	Adenosine diphosphate
AIF	Apoptosis Inducing Factor
ANT	Adenine nucleotide transporter
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
ARE	Antioxidant responsive element
Bcl-2	B cell CLL/lymphoma 2
BH	Bcl-2 homology domains
BIR	Baculovirus IAP Repeat
BIRC	BIR-containing protein
bZIP	Basic-leucine zipper domain
CAD	Caspase-activated DNase
CAK	CDK-activating kinase
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruiting domains
Caspase	Cysteine-aspartic protease
CBP	CREB-binding protein
CDK	Cyclin-dependent protein kinases
Cip/Kip	CDK inhibitory proteins/kinase inhibitory protein
CKI	Cyclin-kinase inhibitors
CNC	Cap N' Collar
CNS	Central nervous system
Cytb5R	NADH-cytochrome b_5 reductase
DED	Death Effector Domain
DEM	Diethylmaleate
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco´s modified essential medium
DMSO	Dimethylsulfoxyde
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EndoG	Endonuclease G
EpRE	Electrophile-responsive element
ER	Endoplasmic Reticulum
ERK1/2	Extracellular signal-regulated kinase

ES cells	Embryonic stem cells
FAD	Oxidized form of flavin adenine dinucleotide
FADD	Fas-associated protein with death domain
$FADH_2$	Reduced form of flavin adenine dinucleotide
$FMNH_2$	Reduced form of flavin mononucleotide
FMN	Oxidized form of flavin mononucleotide
g-GCS	g-glutamylcysteine synthetase
Gpx	Glutahione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferases
HCC	Hepatocellular carcinoma
HO-1	Heme oxigenase-1
Hsp	Heat shock protein
Htr	High-temperature requirement family
IAP	Apoptosis inhibitor
IBM	IAP binding motif
IMM	Inner mitochondrial Membrane
IMS	Intermembrane space
ING	Inhibitor of growth family
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
KO	Knockout
MAC	Mitochondrial apoptosis-induced channel
MAPK	Mitogen-activated protein kinase
MEFs	Murine embryonic fibroblasts
MPT	Mitochondrial permeability transition
MsrA	Methionine sulfoxide reductase A
MTT	Diphenyltetrazolium bromide tetrazolium
NAC	N-acetyl cisteine
NAD^+	Oxidized form of nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
$NADP^+$	Oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NQO1	NAD(P)H-quinone oxidoreductase 1
NLS	Nuclear localization signal

NO	Nitric oxide
NOD	Nucleotide bound and oligomerization domain
Nrf2	Nuclear factor erythroid 2 related factor, NF-E2-related factor 2
ODC	Ornithine decarboxylase
OMM	Outer mitochondrial membrane
OMMP	Outer mitochondrial membrane permeabilization
PARP-1	poly(ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PERK	Pancreatic endoplasmic reticulum kinase
PGC1	Peroxisome proliferator-activated receptor γ coactivator 1
PhGpx	Phospholipid hydroperoxide glutathione peroxidase
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
pRb	Retinoblastoma protein
PRC	PGC related coactivator
Prx	Peroxiredoxin
PTPC	Permeability transition pore complex
RIPA	Radioimmunoprecipitation assay
RMCP	Redox model of cell proliferation
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of the mean
Smac/DIABLO	Second mitochondria-derived activator of caspases or direct IAP binding protein with low pI
Small Maf	Small musculoaponeurotic fibrosarcoma protein
SOD	Superoxide dismutase
tBHQ	Tert-buthylhydroquinone
TCA	Trichloroacetic acid
TNFR	Tumor necrosis factor receptor
Topo IIα	Topoisomerase II-α
Trx	Thioredoxin
VDAC	Voltage dependent anion channels
Xiap	X-linked inhibitor of Apoptosis
XRE	Xenobiotic responsive element

Abstract

1. Introduction

To neutralize oxidative stress insults, cells have acquire complex defense mechanisms which are induced in a highly coordinated way. This coordinated response is regulated through a *cis*-regulatory element, called the antioxidant responsive element (ARE) or electrophile-responsive element (EpRE), located within the regulatory region of target genes (Friling, Bensimon et al. 1990, Rushmore, Morton et al. 1991). Nrf2 (Nuclear factor erythroid 2 related factor, NF-E2-related factor 2) (Moi, Chan et al. 1994) has been identified as the major regulator of ARE-mediated gene expression (Venugopal and Jaiswal 1996, Itoh, Chiba et al. 1997, Ishii, Itoh et al. 2000).

Nrf2 is a Cap N' Collar (CNC) transcription factor, which constitutes a unique subset within the bZIP (basic-leucine zipper domain) transcription factors family (Mathers, Fraser et al. 2004). Under normal conditions, Nrf2 is constantly degraded with a half-life of less than 20 minutes (Katoh, Iida et al. 2005) and thus, degradation of Nrf2 serves as a regulatory control. Nrf2 is bound to a Keap1 dimer in the cytoplasm that suppresses Nrf2-dependent transcriptional activity (Itoh, Wakabayashi et al. 1999). A two-site substrate recognition hinge-and-latch model was proposed to be the key regulatory mechanism. In this model, a Keap1 homodimer recruits Nrf2 *via* the evolutionarily conserved low-affinity DLG and high-affinity ETGE motifs within the regulatory Neh2 domain of Nrf2 (Tong, Katoh et al. 2006, Tong, Kobayashi et al. 2006, Tong, Padmanabhan et al. 2007). Keap1 binds to the actin cytoskeleton and traps Nrf2 in the cytoplasm preventing the Nrf2 nuclear translocation and its subsequent activity (Kang, Kobayashi et al. 2004). Besides its role in sequestering Nrf2 at the cytoplasm, Keap1 also inhibits Nrf2 function through one additional mechanism by acting as an adaptor to allow the interaction between Nrf2 and the cullin3-based E3 ubiquitin ligase complex 2. The formation of this complex triggers the ubiquitylation and the subsequent turnover of Nrf2 by the proteasome (Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004). Under stress conditions Nrf2 is released from Keap1. This dissociation occurs by two different mechanisms: direct attack by electrophiles or ROS, or indirect modifications such as phosphorylation.

Nrf2 has been considered as a proto-oncogene (Wang, Sun et al. 2008, Suzuki, Motohashi et al. 2013) because uncontrolled activation of Nrf2 may allow the survival of potentially dangerous damaged cells leading to oncogenesis and chemoresistance. However, Nrf2 has been also recognized as a tumor suppressor gene (Martin-Montalvo, Villalba et al. 2011) and activation of Nrf2 and its downstream targets may constitute a potentially valuable

therapeutic strategy to treat oxidative stress-induced diseases, including cancer (Suzuki, Motohashi et al. 2013)

An important connection between Nrf2 activity and apoptotic pathways is the Nrf2-mediated regulation of proteins belonging to the Bcl-2 family, like Bcl-2 and Bcl-xL (Chen, Li et al. 2012, Niture and Jaiswal 2012, Niture and Jaiswal 2013). Previous investigations have demonstrated that the expression of antiapoptotic Bcl-2 and Bcl-xL proteins is under Nrf2 direct transcriptional control because of the presence of an ARE in the regulatory regions of the corresponding gene (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Stabilization of Nrf2 in the nucleus also increased the levels of antiapoptotic proteins Bcl-2 and Bcl-xL and resulted in cardioprotective effects in mice subjected to myocardial ischemia/reperfusion (Calvert, Jha et al. 2009). In the context of aging, it has been shown that the impact of Nrf2 deletion on skeletal muscle is increased in aged animals, resulting in higher expression of different apoptotic markers such as caspases-3 and -9, Bax, Bad, and AIF (Miller, Gounder et al. 2012).

Nrf2 regulates a subset of drug metabolizing enzymes, such as NAD(P)H-quinone oxidoreductase 1 (NQO1) (Rushmore, Morton et al. 1991). NQO1 is a flavoprotein that catalyzes the two-electron reduction of quinones and quinoid compounds to hydroquinones, using either NADH or NADPH as the electron donor (Li, Bianchet et al. 1995). The study of NQO1 inhibition effects has been extended to the development of cell lines and organisms that genetically lack *NQO1*. From the discovery of NQO1, this enzyme has shown its functional versatility, being an excellent example of economy in the cell physiology (Dinkova-Kostova and Talalay 2010). NQO1 has a role in detoxification which is related with its quinone reduction function (Joseph, Long et al. 2000), as an *in vitro* $O_2^{\cdot-}$ scavenger (Siegel, Gustafson et al. 2004), as a bioactivator enzyme (Cadenas 1995), as antioxidant enzyme because of the reduction of coenzyme Q or α-tocopherolylquinone (Bello, Kagan et al. 2003) and also interacting with a number of proteins as p53, thus regulating their stability (Asher, Lotem et al. 2002).

A mutation in NQO1 that has been denoted as the *NQO1*2* genetic polymorphism results in the absence of NQO1 activity. Some of the cell lines used in research which are homozygous for the *NQO1*2* polymorphism include BE and Caco-2 of colon carcinoma, MDA-MB-486 and MDA-MB-231 of breast cancer and NCI-H1570 and NCI-H596 of lung cancer (reviewed by (Ross and Siegel 2004)). The presence of *NQO1*2* polymorphism is a robust predictive factor in breast cancer (Fagerholm, Hofstetter et al. 2008). For the hepatocellular carcinoma it has been suggested as a very valuable factor for the prognosis of the evolution of this cancer (Chiu, Ko et al. 2009). This association may be of great importance if we consider

that 4% of Caucasians and approximately 20% of the Asians are homozygous for *NQO1*2* (Ross and Siegel 2004).

2. Main objective

The aim of this study was to analyze the effect of the lack of the transcription factor Nrf2 or its downstream antioxidant target enzyme NQO1 on growth, apoptotic signaling and proliferation pathways in MEFs. For this purpose, several apoptotic and proliferative markers were analyzed and some functional characterizations were also performed.

3. Materials and Methods

Wild-type (Wt), Nrf2KO and NQO1KO mice were maintained at the Gerontology Research Center, National Institutes of Health (Baltimore, MD, USA). MEFs were obtained from fetuses of the three genotypes at day 13 post-coitum. Pregnant female mice were sacrificed by cervical dislocation. Passages of cell cultures were carried out according to the procedure for obtaining the 3T3 cell line (Todaro and Green 1963).

Viability of the cells was assessed by two methods: The trypan blue-exclusion assay by counting the cells with a haemocytometer, a test used to determine the number of viable cells present in a cell suspension, and also by the MTT viability assay. In the latter diphenyltetrazolium bromide tetrazolium (MTT) is converted into a purple colored formazan product with an absorbance maximum near 570 nm, whereas nonviable cells lose the ability to convert MTT into formazan.

Proliferation of the cells was measured by an assay that evaluates the rate of incorporation of the radiolabeled DNA precursor ^{3}H-thymidine into the replication strands of DNA produced during S phase of the cell division cycle.

In order to assess the migration capacity of the cells the wound healing assay was performed. Using a sterilized 200 µl tip we scratched several wounds in cells previously grown to 90% of confluence. Pictures at time 0 and after 8 hours were taken in the same area. The healing process was quantified by ImageJ.

The cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) was used to measure the levels of intracellular peroxides. Samples were analyzed is an EPICS XL flow cytometer (Beckmann Coulter). For measuring mitochondrial membrane

potential we used the lipophilic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1 probe), also by flow cytometry.

Ultrastructural analysis of mitochondria was performed by using electron microscopy. Accumulation of spontaneous DNA lesions in MEFs of the different genotypes was investigated by immunostaining for phosphorylated histone H2A.X (γH2A.X), a marker of DNA double-strand breaks (DSBs).

For the preparation of whole cell extracts MEFs were disrupted by homogenization in RIPA buffer. Prior to extraction, cells were centrifuged at 500 x *g* for 5 min, washed with PBS and centrifuged again under the same conditions. The cell pellet was resuspended in RIPA buffer and, after agitation, the suspension was centrifuged at 10,000 x *g* for 15 minutes.

In order to obtain cytosolic and nuclear fractions, the cell pellets were resuspended in a hypotonic buffer and incubated for 15 minutes. After a brief vortexing followed by addition of NP-40, the cell extracts were centrifuged at 2,800 x *g* in a tabletop microfuge. Supernatants so obtained constitute crude cytosolic fractions, which were centrifuged again at 135,000 x *g* in a Beckman Optima TLX ultracentrifuge for further purification. The pellets from the 2,800 x *g* centrifugation, containing the nuclei, were resuspended in a high-salt buffer. Nuclear extracts were recovered after centrifugation for 10 minutes at 10,000 x *g* in a tabletop microfuge.

Proteolytic activities of caspases-8, -9 and -3 were determined in a fluorometric assay, either in the presence or in the absence of specific inhibitors. Fluorescence signals were then registered at wavelengths of 380 nm (excitation) and 460 nm (emission). DNA fragmentation (apoptotic index) was quantified using the ELISA kit developed by Roche Diagnostics, which is based on the measurement of the amount of cytosolic mono- and oligonucleosomes derived from chromatin fragmentation.

For immunodetection of cell death-related proteins, mitochondrial dynamics, MAPK and Akt, an approximate amount of 50 μg protein was separated by SDS-PAGE and then transferred to nitrocellulose membranes. Blots were stained with Ponceau S for visualization and quantification of protein lanes. The majority of polypeptides were measured in whole homogenates, while AIF was measured in nuclear fractions and cytochrome *c* in cytosolic fractions. Horseradish peroxidase activity was used to reveal binding sites by enhanced chemiluminescence.

MEFs were injected in nude mice for investigating their tumorigenic potential. B10F16 cancer cells from mouse skin melanoma were used as a positive control. 26 days after the injections animals were sacrificed and the tumors excised. To study the histopathology of teratomas, the tumors were fixed and embedded in paraffin following routine histological procedures. Both procedures were carried out in the National Institute of Health (Baltimore, MD). We used haematoxylin-eosin for staining of the teratoma sections.

For studies involving tissues, five months old male wild-type (Wt), NQO1KO and Nrf2KO mice on C57BL/6 background were sacrificed by cervical dislocation at National Institute on Aging (NIH). The organs were excised and quickly frozen in liquid nitrogen. 50 mg of tissues were weighted and, in order to obtain total homogenates, the tissue samples were then lysed in RIPA buffer. To isolate the cytosolic and the nuclear fractions from liver the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, USA) was used in accordance with the manufacturer's instructions. The obtained fractions were used for western blotting followed by immunodetection and for determination of apoptotic index by ELISA.

4. Results and Discussion

4.1. Nrf2

Nrf2 has been recently shown to control the expression of several antiapoptotic proteins, as Bcl-2 and Bcl-xL (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Thus, we aimed to demonstrate if genetic deletion of Nrf2 actually increases apoptosis in the absence of pro-apoptotic stimuli. We found out that level of basal apoptosis was increased in Nrf2KO MEFs and also in two mitotic tissues of Nrf2KO mice, lung and liver. A previous work in the field has reported that the inhibition of Nrf2-dependent transcription by gene silencing is linked to the enhancement of etoposide and radiation-induced apoptosis in mouse and human hepatic tumor cells (Niture and Jaiswal 2012). In addition, Nrf2KO mice show higher levels of several pro-apoptotic factors in skeletal muscle from aged mice (Miller, Gounder et al. 2012, Narasimhan, Hong et al. 2014).

The significant decrease of Bcl-2 levels and Bcl-2/Bax ratio observed in Nrf2KO MEFs is consistent with an enhancement of the mitochondrial pro-apoptotic pathway in these cells. Genetic deletion of Nrf2 also increases cytosolic cytochrome *c* and nuclear AIF levels in mitotic cells. Furthermore the increase of nuclear AIF observed in Nrf2KO MEFs agrees with the AIF increase in skeletal muscle from aged Nrf2KO mice (Miller, Gounder et al. 2012). In addition, we found that Apaf-1 levels were significantly increased in Nrf2KO MEFs. Since,

under these conditions caspases of the mitochondrial pathway were not activated, our results support that mitochondrial apoptotic signaling is blunted at the level of caspase cascade activation in cells lacking Nrf2. It is thus very likely that the increase of DNA fragmentation observed in Nrf2KO MEFs could be mediated by increased nuclear levels of AIF, as it has been demonstrated that AIF is able to induce chromatin condensation and fragmentation in a caspase-independent pathway (Susin, Lorenzo et al. 1999).

One possibility to explain the lack of caspase-3 activation would involve the participation of the Inhibitors of Apoptotic Proteases (IAPs). One of the main IAPs is the X-chromosome linked Inhibitor of Apoptotic Proteases (XIAP), known to behave as a potent inhibitor of caspase-9 and effector caspases (Jost, Grabow et al. 2009). We found no change of XIAP levels among wild-type and Nrf2KO MEFs. However, when cells were treated with BV6, a Smac mimetic and XIAP inhibitor, wild-type and Nrf2KO MEFs behaved in a different way because cell death was readily induced in Nrf2KO but not in wild-type MEFs. The induction of cell death in Nrf2KO MEFs when treated with the Smac mimetic can be due to the fact that MEFs lacking Nrf2 have a very high content of pro-apoptotic factors although the intrinsic caspase pathway is blunted, most likely because XIAP levels in these cells are enough to block the caspase cascade at the caspase-3 level. In addition, higher levels of procaspase-9 prevents caspase-3 activation.

Although ERK1/2 and p38 MAPKs were activated in Nrf2KO MEFs, they were unlikely to participate in regulating basal apoptosis levels in these cells, because DNA fragmentation was insensitive to pretreatment with well characterized pharmacological inhibitors of MAPK pathways. However, by using p38 inhibitors SB208530 and PD169315 we demonstrate that p38 is clearly a negative effector of Bcl-2 function in wild-type MEFs through the inhibition of Nrf2 transcriptional activity and the phosphorylation of Bcl-2. However, this anti-apoptotic protein becomes independent on p38 activity in Nrf2KO MEFs.

Monoamine oxidase (MAO) activity has been related with mitochondrial permeabilization, which can be abolished by inhibitors of its two isoforms, MAO-A and -B, in rat liver mitochondria (Marcocci, De Marchi et al. 2002). Interestingly, Nrf2 has been described to downregulate MAO-B mRNA levels in a transcriptomic study (Reddy, Kleeberger et al. 2007). Taken into consideration these premises, we hypothesized that MAO-B could be involved, at least in part, in the increased mitochondrial permeabilization observed in Nrf2KO MEFs. In accordance, the MAO-B inhibitor deprenyl decreased DNA fragmentation in wild-type and in Nrf2KO MEFs.

In our ultrastructural analysis focused on mitochondria we have found that mitochondrial area was smaller in Nrf2KO MEFs when compared with their wild-type counterparts. However, at a cellular level mitochondria were more abundant and there was a higher surface of cytoplasm occupied by these organelles. This suggests that the higher number of mitochondria in Nrf2KO MEFs could be a consequence of alterations in the fusion/fission process. Measurements of fusion and fission marker proteins suggested a decrease in fusion with no differences in fission markers. Lower levels of the fusion protein Mfn2 are also compatible with a study that describe that silencing of Mfn1/2 causes the generation of shorter mitochondria and the increase of apoptotic levels (Sugioka, Shimizu et al. 2004).

4.2. NQO1

Cultures of MEFs lacking NQO1 exhibited longer life span and higher proliferation rates when compared their wild-type counterparts. This is consistent with a role for the lack of NQO1 in tumor progression. Nrf2 signaling has been suggested to lighten the effects of oxidative stress (including some aging-associated diseases like neurodegeneration, chronic inflammation, and cancer both in rodents and humans). In a cellular model of human fibroblasts it has been shown that Nrf2 function is declined in senescence, and also that Nrf2 silencing leads to premature senescence. Moreover, treatment with a Nrf2 inducer results in the enhanced survival of cells following oxidative stress, whereas continuous treatment leads to life span extension of human fibroblasts (Kapeta, Chondrogianni et al. 2010). Moreover, activation of Nrf2 signaling has a pro-longevity effect in *Drosophila megalonaster* males (Sykiotis and Bohmann 2008, Sykiotis and Bohmann 2010). Interestingly, Nrf2-dependent signaling has been also identified as a major determinant of cellular stress resistance in a model of mice with extended longevity (Leiser and Miller 2010). In accordance with the importance of Nrf2 in the life span, we observed a higher nuclear translocation of Nrf2 in NQO1KO MEFs and in liver of NQO1KO mice. The possibility exists that this increase of nuclear Nrf2 has been developed as a hormetic response to compensate the lack of the antioxidant enzyme NQO1.

Surprisingly, in our experimental model we have observed higher levels of basal apoptosis in NQO1KO MEFs. However, concomitance of apoptosis and excessive growth has been also observed in certain types of tumors which present an uncontrolled growth that leads to high levels of apoptosis as well (Hikita, Kodama et al. 2012).

When we analyze different apoptotic markers we found that the increase of the Bcl-2/Bax ratio in NQO1KO MEFs is not consistent with the cytosolic release of cytochrome *c* and the increase of the nuclear AIF in NQO1KO MEFs. For this reason we also analyze the levels of

Bid, another pro-apoptotic protein of the Bcl-2 family. In our hands NQO1KO MEFs exhibited higher levels of full-length Bid. However, despite the enhancement of caspase-8 activation, no more truncated Bid (tBid) was generated in MEFs lacking NQO1. It has been described that, even though the full activity is acquired after Bid cleavage, full-length Bid also contains some pro-apoptotic function (Billen, Shamas-Din et al. 2008). Mitochondrial pathway was enhanced with activation of caspase-9 and -3, although cells might also undergo caspase-independent apoptosis through AIF. However, despite the high activation of caspase-8, the extrinsic pathway initiator, the connection between extrinsic and intrinsic pathways is apparently blocked in NQO1KO MEFs. However, it is known that caspase-8 plays several cellular roles in vertebrates, not only mediating extrinsic apoptosis, but also regulating endosomal trafficking, autophagy and enhancing cell migration and adhesion (Stupack 2013). This can explain why normal or high levels of this caspase are found in several tumors (Barbero, Barila et al. 2008). High levels of active caspase-8 correlated with a high healing capacity of NQO1KO cells.

Akt is a protein involved in a pathway related to proliferation and survival (Altomare and Khaled 2012). The expression of Nrf2 in proliferating cells directs the enhancement of anabolic metabolism, maintains redox homeostasis and further promotes the activation of PI3K–Akt signaling, suggesting the presence of a positive feedback loop between Nrf2 and the PI3K–Akt pathway in proliferating cells (Mitsuishi, Motohashi et al. 2012). In the case of NQO1KO cells, we have observed higher levels of phospho-Akt and Nrf2 nuclear translocation. The increase of these two proteins might confer growth advantage to these cells. This is consistent with the extended long-term culture lifespan in MEFs lacking NQO1 as well as a higher proliferation rate. It is known that active Akt phosphorylates apoptosis signaling kinase 1 (Ask1) at Serine 83, which attenuates Ask1 activity and promotes cell survival. Ask1 transduces stress signals to the pro-apoptotic c-Jun N-terminal kinase (JNK) and p38 MAP kinase cascades (Kim, Khursigara et al. 2001). These regulation pathway is consistent with our results with NQO1KO MEFs., which exhibited higher levels of active Akt and decreased phosphorylated p38 and JNK1.

Their low levels of activated (phosphorylated) p38 may be another factor contributing to the phenotype of NQO1KO MEFs . Dolado and colleagues characterized p38 as a ROS sensor and also highlighted its importance in the prevention of tumorigenesis in oncogene-transfected MEFs. These authors evidenced that MEFs lacking p38 were more prone to transformation by H-Ras (Dolado, Swat et al. 2007). The low levels of P-p38 in NQO1KO MEFs could be a mechanism to prevent apoptosis, as an attemp to uncouple the p38-dependent pathway. In addition to its role in apoptosis, a necessity for p38 has been also

described for contact inhibition in a p38KO MEFs model (Faust, Dolado et al. 2005). We speculate with the idea that the lack of p38 can be partly responsible of the high cell saturation density of the NQO1KO cells. Similarly, previous results obtained by different groups (including ours) have demonstrated that the NQO1 expression depends on the cell culture confluency in normal adherent and tumor cells. NQO1 was increased at high cell density in BALB/c 3T3 cells (Schlager, Hoerl et al. 1993), human osteoblasts (Collin, Lomri et al. 2001) and HeLa cells (Bello, Gomez-Diaz et al. 2001, Bello, Gomez-Diaz et al. 2004).

Injection of NQO1KO MEFs into nude mice led to the development of teratomes, whereas wild-type MEFs were inactive. When we examined the slices of these teratomas in the light microscope, several mesodermic cells and tissues (fibroblasts-like cells, white and brown adipose tissue, muscle) were observed, highlighting the proliferative and differentiation potential of MEFs lacking NQO1.

NQO1KO MEFs presented smaller mitochondria when compared to their wild-type counterparts. However, as also found in Nrf2KO cells, these organelles were more abundant and occupied a higher surface proportion of cytoplasm. Alterations in the fusion/fission process might explain the mitochondrial structural differences between both genotypes. We have observed a decrease in two fusion proteins, Opa1 and Mfn2, but no differences in fission protein markers. This is consistent with a study in which NQO1 was suggested to be needed for maintaining mitochondrial integrity (Kwon, Han et al. 2012).

Taken together, our results indicate that, although Nrf2 and NQO1 participate in the same antioxidant signaling axis (Rushmore, Morton et al. 1991), the deletion of either Nrf2 or NQO1 in MEFs generates very different cellular phenotypes.

Resumen

1. Introducción

El factor-2 relacionado con el factor nuclear E2 (Nrf2) es un factor de transcripción esencial para la regulación de muchas enzimas antioxidantes y destoxificadoras de fase II inducidas por estrés (Moi, Chan et al. 1994). Esta respuesta es muy compleja y se encuentra coordinada mediante un elemento *cis* llamado *elemento de respuesta antioxidante* (ARE) o *elemento de respuesta a electrofílicos* (EpRE), que se localiza en la región reguladora de los genes diana correspondientes (Friling, Bensimon et al. 1990, Rushmore, Morton et al. 1991). Nrf2 es el principal factor de transcripción capaz de aumentar la transactivación de genes que se expresan de forma regulada por la secuencia ARE (Venugopal and Jaiswal 1996, Itoh, Chiba et al. 1997, Ishii, Itoh et al. 2000).

Nrf2 es un factor de transcripción que pertenece al grupo de los factores CNC (Cap N' Collar). Éstos constituyen un subgrupo dentro de la familia de factores de transcripción que poseen una cremallera de leucina básica (bZIP) (Mathers, Fraser et al. 2004). En condiciones normales Nrf2 se está degradando de forma continua y presenta una vida media de menos de 20 minutos (Katoh, Iida et al. 2005). Por tanto, la degradación forma parte de la regulación de la actividad Nrf2. Nrf2 se encuentra unido a un dímero de Keap1 en el citoplasma que, de esta forma, reprime su actividad transcripcional (Itoh, Wakabayashi et al. 1999). La interacción entre Nrf2 y su represor Keap1 ocurre mediante un mecanismo de llamado de pestillo y bisagra (*hinge-and-latch*). En este modelo, se produce una doble unión mediante dos motivos, uno de alta afinidad (ETGE) y otro de baja afinidad (DLG). Ambos se encuentran en el dominio Neh2 de Nrf2 (Tong, Katoh et al. 2006, Tong, Kobayashi et al. 2006, Tong, Padmanabhan et al. 2007). Keap1 se encuentra unido a su vez al citoesqueleto de actina para retener a Nrf2 en el citoplasma y evitar que se transloque al núcleo, donde Nrf2 ejerce su acción (Kang, Kobayashi et al. 2004). Además de secuestrar Nrf2 en el citoplasma, Keap1 también se une a cullin-3 formándose el complejo 2 de ubiquitina ligasa E3. Este complejo es capaz de ubiquitinar Nrf2 y marcarlo para su degradación en el proteasoma (Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004). En condiciones de estrés Nrf2 es liberado de Keap1. Esta disociación es posible gracias a dos mecanismos diferentes, por modificación directa mediante ROS y sustancias electrofílicas, o por modificaciones como la fosforilación.

Nrf2 es considerado un proto-oncogén (Wang, Sun et al. 2008, Suzuki, Motohashi et al. 2013) debido a que su activación crónica favorece la supervivencia de células potencialmente dañadas y promueve, por tanto, la oncogénesis así como los procesos de quimioresistencia. Sin embargo, Nrf2 es considerado también un gen supresor de tumores

(Martin-Montalvo, Villalba et al. 2011) siendo la activación de Nrf2 y sus genes diana una estrategia terapéutica para prevenir enfermedades relacionadas con el estrés oxidativo entre las que también se encuentra el cáncer (Suzuki, Motohashi et al. 2013).

Existe también una regulación de Nrf2 sobre algunas proteínas de la familia Bcl-2, como son Bcl-2 y Bcl-xL (Chen, Li et al. 2012, Niture and Jaiswal 2012, Niture and Jaiswal 2013). Hay estudios que demuestran que la expresión de las proteína antiapoptóticas Bcl-2 y Bcl-xL está regulada directamente por Nrf2 debido a la presencia de un ARE en la región reguladora de los genes correspondientes (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Una mayor estabilización de Nrf2 y su consecuente translocación al núcleo provocan un aumento de Bcl-2 y Bcl-xL teniendo un efecto cardioprotector en ratones que han sufrido isquemia/reperfusión (Calvert, Jha et al. 2009). En relación con la edad, la deleción de Nrf2 en músculo esquelético provoca una mayor expresión de varios marcadores de apoptosis como caspasa-3 y -9, Bax, Bad y AIF en animales viejos (Miller, Gounder et al. 2012).

Nrf2 regula la transcripción de gran grupo de enzimas antioxidantes y destoxificadoras entre las que se encuentra la NAD(P)H quinona oxidorreductasa 1 (NQO1) (Rushmore, Morton et al. 1991). NQO1 es una flavoenzima que cataliza la reducción de compuestos quinónicos a hidroquinonas mediante una reacción de dos electrones, utilizando tanto NADH como NADPH como donador de electrones (Li, Bianchet et al. 1995). El estudio de la inhibición de esta enzima se ha extendido al desarrollo de líneas celulares y organismos que carecen del gen NQO1. Desde su descubrimiento, esta enzima se ha mostrado como un ejemplo de versatilidad y economía en la fisiología celular (Dinkova-Kostova and Talalay 2010). De hecho NQO1 tiene un papel en la destoxificación estrechamente relacionado con su función de reducción de quinonas (Joseph, Long et al. 2000), también funciona como un *scavenger* de $O_{2^{-}}$ (Siegel, Gustafson et al. 2004) y como enzima bioactivadora (Cadenas 1995). Además, tiene un importante papel como antioxidante al reducir el coenzima Q o la α-tocoferilquinona (Bello, Kagan et al. 2003), así como interaccionando con varias proteínas entre las que se encuentra p53, sobre la que actúa regulando su estabilidad (Asher, Lotem et al. 2002).

Un polimorfismo de NQO1 denominado como *NQO1*2* resulta en la total carencia de actividad y polipéptido NQO1. Algunas de las líneas celulares ampliamente utilizadas en investigación, como las BE o las Caco-2 de carcinoma de colon, MDA-MB-864 y MDA-MB-231 de cáncer de mama así como las NCl-H1570 y NCl-H596 de cáncer de pulmón, poseen dicho polimorfismo (revisado por (Ross and Siegel 2004)). De hecho, el polimorfismo *NQO1*2* es un factor predictivo en cáncer de mama (Fagerholm, Hofstetter et al. 2008).

Asimismo en el carcinoma hepatocelular es un factor a considerar en la prognosis de la evolución del cáncer (Chiu, Ko et al. 2009). La relación entre estas patologías y el polimorfismo es de gran importancia teniendo en cuenta que el 4% de los caucásicos y aproximadamente un 20% de los asiáticos son homocigotos para *NQO1*2* (Ross and Siegel 2004).

2. Objetivo principal

El principal objetivo de este estudio es el análisis del efecto de la falta del factor de transcripción Nrf2 o de su diana NQO1 en el crecimiento, apoptosis y proliferación de MEFs. Con este objetivo, se midieron varios marcadores de distintas vías de señalización apoptótica y de proliferación. Además, fueron también llevados a cabo algunos estudios funcionales.

3. Materiales y métodos

Ratones silvestres (Wt), Nrf2KO y NQO1KO fueron mantenidos en el Gerontology Research Center, National Institutes of Health (Baltimore, MD, USA). Los MEFs se obtuvieron a partir de fetos diseccionados de los tres genotipos, obtenidos a partir de ratones hembra en el día 13 post coito. Las hembras preñadas se sacrificaron por dislocación cervical. Las líneas inmortalizadas de MEFs se generaron siguiendo el protocolo de subcultivos para las células 3T3 (Todaro and Green 1963).

La viabilidad celular se estudió mediante dos métodos: a través del método de exclusión del azul tripán con el contaje de células con un hemocitómetro, un test que se usa para conocer el número de células viables de una suspensión, y también mediante ensayo de MTT. En este último, el bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) se convierte en un compuesto formazán de color azul, que tiene una absorbancia cercana a 570 nm; perdiendo las células no viables la capacidad de convertir el MTT a formazán.

La proliferación de las células se midió por un ensayo de incorporación de timidina. Para ello se utilizó un precursor de ADN con marcaje radiactivo (timidina tritiada). Este precursor se incorpora durante la replicación del ADN que tiene lugar en la fase S del ciclo celular.

Con el objetivo de evaluar la capacidad migratoria de las células se llevó a cabo un ensayo de curación de herida. Para ello utilizamos una punta de pipeta de 200 µl con la que

realizamos una herida en un cultivo de células al 90% de confluencia. Se hicieron fotos a tiempo cero y después de 8 horas, y se analizó el porcentaje de curación mediante el programa ImageJ.

Para medir los niveles de peróxidos intracelulares, se utilizó la sonda diacetato de 2´,7´-diclorodihidrofluoresceina (DCFH-DA). Las muestras fueron analizadas en el citómetro de flujo EPICs XL (Beckmann Coulter). El potencial mitocondrial se determinó con el fluorocromo lipofílico 5,5′,6,6′-tetracloro-1,1′,3,3′-tetraetilbenzimidazol-ioduro de carbocianina (JC-1) mediante citometría de flujo.

El análisis ultraestructural de la mitocondria se llevó a cabo mediante microscopía electrónica. La acumulación de lesiones en el ADN de MEFs de los diferentes genotipos se estudió mediante inmunocitoquímica midiendo la histona fosforilada H2A.X (γH2A.X), un marcador de roturas de la doble cadena de ADN.

Los extractos totales de MEFs se prepararon utilizando RIPA buffer. Antes de la extracción las células se centrifugaron a 500 x *g* durante 5 minutos, se lavaron con PBS y se volvieron a centrifugar. Las pellas celulares se resuspendieron en RIPA buffer y, tras ser agitadas, se centrifugaron a 10,000 x *g* durante 15 minutos para obtener los extractos en los sobrenadantes.

Para la obtención de fracciones citosólicas y nucleares, las pellas de células se resuspendieron en un tampón hipotónico en el cual fueron incubadas durante 15 minutos. Después de una agitación y la posterior adición del detergente NP-40, los extractos se centrifugaron a 2,800 x *g*. Los sobrenandantes se volvieron a centrifugar a 135,000 x *g* en una ultracentrífuga Beckman Optima TLX para la obtención de citosoles purificados. Las pellas obtenidas en la centrifugación a 2,800 x *g*, que contienen los núcleos, se resuspendieron en un tampón con un alto contenido en sales. Las fracciones nucleares fueron recogidas a partir de los sobrenadantes obtenidos tras una centrifugación de 10 minutos a 10,000 x *g*.

Las actividades proteolíticas de las caspasas -8, -9 y -3 fueron determinadas mediante un ensayo fluorimétrico en presencia y en ausencia de inhibidores específicos. La señal de fluorescencia fue detectada a las longitudes de onda de 380 nm (excitación) y 460 nm (emisión). La fragmentación del ADN (índice apoptótico) fue cuantificada utilizando el *kit* de ELISA desarrollado por Roche Diagnostics, que se basa en la detección de fragmentos mono- y oligonucleosomales derivados de la rotura de la cromatina.

Para la inmunodetección de proteínas relacionadas con la muerte celular, dinámica mitocondrial, rutas MAPK y Akt, una cantidad aproximada de 50 µg de proteína fue separada mediante SDS-PAGE. A continuación, se transfirió a membranas de nitrocelulosa. Dichas membranas fueron teñidas con Ponceau S para visualizar y cuantificar las proteínas transferidas. La mayoría de las proteínas fueron medidas en homogenados totales, mientras que AIF fue medido en las fracciones nucleares y citocromo *c* en las citosólicas. La unión de los anticuerpos específicos fue revelada mediante quimioluminiscencia asociada a actividad peroxidasa.

Para investigar el potencial tumorigénico de los MEFs, éstos fueron inyectados en ratones inmunodeficientes. La línea celular B10F16 de melanoma de piel se usó como control positivo en la generación de tumores. Después de 26 días los ratones se sacrificaron y los tumores fueron aislados. Para el estudio de su histopatología, éstos se fijaron y se incluyeron en parafina. Los dos procesos se llevaron a cabo en el National Institutes of Health (Baltimore, MD). La técnica de hematoxilina y eosina se utilizó para la tinción y posterior observación de los cortes de teratoma.

Para los estudios con tejidos, ratones macho de 5 meses de la estirpe C57BL/6 Wt, NQO1KO y Nrf2KO se sacrificaron por dislocación cervical en el National Institutes of Health (Baltimore, MD). Se extrajeron los órganos y se congelaron rápidamente en nitrógeno líquido. 50 mg de tejido fueron pesados y para obtener homogenados totales, que se lisaron con RIPA buffer. Para aislar fracciones citosólicas y nucleares de hígado se utilizó el NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, USA) siguiendo las instrucciones del fabricante. Dichas fracciones se usaron para *western blot* y la posterior inmunodetección, así como para la determinación del índice apoptótico (fragmentación de DNA) mediante ELISA.

4. Resultados y Discusión

4.1. Nrf2

Se conoce que Nrf2 controla la expresión de algunas proteínas antiapoptóticas como Bcl-2 and Bcl-xL (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Por tanto, quisimos comprobar si la deleción genética de Nrf2 aumenta los niveles de apoptosis basal. Al medir los niveles basales de apoptosis en MEFs Nrf2KO y en dos tejidos mitóticos como son pulmón e hígado, nos encontramos que estaban incrementados significativamente en los KOs. Un estudio previo había demostrado que la inhibición de Nrf2 mediante silenciamiento provocaba una mayor respuesta apoptótica en células de tumor hepático de ratón y

humanas tras tratamiento con etopósido y radiación (Niture and Jaiswal 2012). Además, los ratones viejos Nrf2KO muestran unos niveles mayores de varios factores pro-apoptóticos en músculo esquelético (Miller, Gounder et al. 2012, Narasimhan, Hong et al. 2014).

El descenso de los niveles de Bcl-2 y del ratio Bcl-2/Bax en MEFs Nrf2KO es coherente con una mayor activación de la vía mitocondrial de la apoptosis. Así, la deleción genética de Nrf2 incrementó además los niveles de citocromo *c* citosólico y de AIF nuclear. El incremento de AIF nuclear encontrado en este estudio es consistente con el aumento de AIF observado en el músculo esquelético de ratones Nrf2KO (Miller, Gounder et al. 2012). Los niveles de Apaf-1 también resultaron incrementados en los MEFs Nrf2KO. Ya que las caspasas de la ruta mitocondrial no aparecieron activadas, nuestros resultados apoyan el hecho de que la cascada apoptótica de la ruta mitocondrial parece estar bloqueada en estas células. Es por tanto probable que el incremento de la fragmentación del ADN detectado en las células Nrf2KO se deba a unos niveles altos de AIF, ya que ha sido demostrado que AIF puede inducir la condensación y la fragmentación de la cromatina en una ruta independiente de caspasas (Susin, Lorenzo et al. 1999).

Una posibilidad que explicaría la falta de activación de la caspasa-3 sería la presencia de inhibidores de proteasas apoptóticas (IAPs). Entre ellos, uno de los más importantes es XIAP, el inhibidor ligado al cromosoma X de proteasas apoptóticas, que se sabe es un potente inhibidor de caspasa-9 y caspasas efectoras (Jost, Grabow et al. 2009). Los niveles de XIAP se mantuvieron similares en los MEFs control y en los Nrf2KO. Sin embargo, cuando las células fueron tratadas con BV6, un inhibidor de XIAP, se indujo la muerte celular en los Nrf2KO pero no en los MEFs control. La inducción de la muerte en los MEFs Nrf2KO al ser tratados con el BV6 puede deberse al alto contenido en factores proapoptóticos, aunque la ruta intrínseca estaría bloqueada en condiciones normales, probablemente debido a que los niveles de XIAP de estas células son suficientes para inhibir la cascada de caspasas al nivel de caspasa-3. Además, se conoce que niveles altos de procaspasa-9 pueden también inhibir la activación de caspasa-3 (Malladi, Challa-Malladi et al. 2009).

Aunque las MAKP ERK1/2 y p38 presentaron una mayor activación en los MEFs Nrf2KO, probablemente no participan en la regulación basal de la apoptosis en estas células ya que los niveles de fragmentación de ADN no se vieron afectados con tratamientos con inhibidores farmacológicos bien caracterizados de la ruta de las MAPK. Sin embargo, mediante el uso de estos inhibidores demostramos que p38 es un regulador negativo de la función Bcl-2 en los MEFs control a través de la inhibición de la actividad transcripcional de

Nrf2 y la fosforilación de Bcl-2. Sin embargo, esta proteína anti-apoptótica no depende de la actividad p38 en los MEFs Nrf2KO.

La actividad monoamino oxidasa (MAO) está relacionada con la permeabilización mitocondrial, la cual puede ser abolida mediante el uso de inhibidores de sus dos isoformas MAO-A y MAO-B en mitocondrias de hígado de rata (Marcocci, De Marchi et al. 2002). Es interesante el hecho de que Nrf2 disminuye los niveles de ARNm de MAO-B, como se ha demostrado en un estudio transcriptómico (Reddy, Kleeberger et al. 2007). Teniendo esto en cuenta, nos planteamos que MAO-B podría ser responsable, al menos en parte, del incremento de la permeabilización mitocondrial observada en los MEFs Nrf2KO. De acuerdo con nuestra hipótesis, el inhibidor de MAO-B deprenil disminuyó la fragmentación del ADN en MEFs control y Nrf2KO.

En nuestro análisis ultraestructural centrado en la mitocondria, hemos observado mitocondrias con una menor área en los Nrf2KO MEFs. Sin embargo, éstas fueron más abundantes, existiendo una mayor superficie de citoplasma ocupado por estos orgánulos en las células Nrf2KO. Esto sugiere que el alto número de mitocondrias en los MEFs Nrf2KO podría ser una consecuencia de alteraciones en los procesos de fusión y fisión mitocondrial. Las medidas de los marcadores de fusión y fisión sugieren un descenso en la fusión sin diferencias en los marcadores de fisión. Unos menores niveles de la proteína de fusión Mfn2 son compatibles con los resultados de un estudio previo que describe que el silenciamiento de Mfn1 y 2 genera mitocondrias más pequeñas y un incremento de los niveles de apoptosis (Sugioka, Shimizu et al. 2004).

4.2. NQO1

Los cultivos de MEFs carentes de NQO1 mostraron una mayor longevidad en cultivo y una mayor tasa de proliferación al compararlos con los controles. Esto es consistente con el papel de NQO1 en la progresión tumoral. Se ha sugerido que la ruta de señalización de Nrf2 puede disminuir los efectos del estrés oxidativo (incluyendo algunas patologías asociadas con la edad como son la inflamación, la neurodegeneración o el cáncer). En un modelo celular de fibroblastos humanos se ha mostrado que Nrf2 decae en la senescencia y que el silenciamiento de este factor de transcripción induce una senescencia prematura. Además, el tratamiento con un inductor de Nrf2 aumenta la supervivencia de las células en condiciones de estrés oxidativo, mientras que el tratamiento continuado aumenta la longevidad en fibroblastos humanos (Kapeta, Chondrogianni et al. 2010). De la misma manera, la activación de la señalización mediada por Nrf2 promueve la longevidad en machos de *Drosophila megalonaster* (Sykiotis and Bohmann 2008, Sykiotis and Bohmann

2010). La señalización dependiente de Nrf2 se ha identificado además como determinante para la resistencia a estrés en un modelo de ratones con una mayor longevidad (Leiser and Miller 2010). De acuerdo con la importancia de Nrf2 en la longevidad, hemos observado una mayor translocación de Nrf2 al núcleo en MEFs NQO1KO y en el hígado de ratones NQO1KO. Existe la posibilidad de que este incremento de Nrf2 en el núcleo se haya desarrollado como una respuesta hormética para compensar la falta de la enzima antioxidante NQO1.

Sorprendentemente, también hemos detectado unos niveles más altos de fragmentación del ADN en los MEFs NQO1KO. Sin embargo, la simultaneidad de una mayor apoptosis y un crecimiento exacerbado se ha observado en algunos tipos de tumores, que presentan un crecimiento descontrolado que origina altos niveles de apoptosis (Hikita, Kodama et al. 2012).

Cuando analizamos diferentes marcadores de la apoptosis encontramos que el incremento del ratio Bcl-2/Bax en los MEFs NQO1KO no es consistente con su mayor liberación de citocromo *c* al citosol y sus niveles más altos de AIF nuclear. Por esta razón también analizamos los niveles de Bid, otra proteína pro-apoptótica de la familia Bcl-2. Los MEFs NQO1KO presentaron mayores niveles de Bid. Sin embargo, a pesar de la mayor activación de caspasa-8, no se encontraron mayores niveles de Bid truncado (tBid). Se ha descrito que, aunque para la adquisición de la actividad total de Bid se necesita que se active proteolíticamente y se genere tBid, Bid también tiene cierta capacidad para inducir apoptosis (Billen, Shamas-Din et al. 2008).

La ruta mitocondrial de la apoptosis estuvo claramente activada con unos niveles mayores de caspasa-9 y -3 procesada, aunque las células también podrían sufrir apoptosis independiente de caspasas mediante AIF. Sin embargo, a pesar de la alta activación de caspasa-8, iniciadora de la vía extrínseca, la conexión de la vía extrínseca e intrínseca se encontró bloqueada en los MEFs NQO1KO. Sin embargo, es bien conocido que la caspasa-8 tiene diferentes funciones en vertebrados y no solamente regula la respuesta apoptótica sino que también regula otros procesos como el tráfico vesicular, autofagia y la migración y adhesión celular (Stupack 2013). Esto puede explicar por qué niveles normales o altos de esta caspasa pueden ser hallados en varios tumores (Barbero, Barila et al. 2008). Altos niveles de caspasa-8 se corresponden tambiéncon la alta capacidad de curación de las células NQO1KO.

Akt es una proteína relacionada con proliferación y supervivencia (Altomare and Khaled 2012). La expresión de Nrf2 activa el metabolismo anabólico, mantiene la homeostasis

rédox y promueve la activación de la ruta PI3K–Akt, sugiriendo una retroalimentación positiva entre Nrf2 y la ruta PI3K–Akt (Mitsuishi, Motohashi et al. 2012). En el caso de las células NQO1KO, hemos observado niveles más altos de fosfo-Akt y translocación nuclear de Nrf2. El incremento de estas dos proteínas puede conferir cierta ventaja de crecimiento a estas células, siendo esto consistente con una extensión de la longevidad en cultivo y unas velocidades de proliferación más elevadas. Se sabe que Akt fosforila a la kinasa señalizadora de la apoptosis (Ask1) en la Serina 83, atenuado su actividad y promoviendo la supervivencia, ya que Ask1 transduce la señal a JNK y p38 (Kim, Khursigara et al. 2001). Esto es consistente con nuestros datos obtenidos en MEFs NQO1KO, los cuales mostraron mayores niveles de Akt y menores de p38 y JNK1.

Los bajos niveles de activación de p38 podrían constituir otro factor que contribuye al fenotipo de los MEFs NQO1KO. Dolado y sus colaboradores caracterizaron la MAPK p38 como un sensor de ROS y destacaron su importancia en la prevención de la tumorigénesis en MEFs transfectados con un oncogén. Así, estos autores mostraron que los MEFs carentes de p38 eran más propensos a la transformación mediante H-Ras (Dolado, Swat et al. 2007). Los bajos niveles de P-p38 observados en los MEFs NQO1KO pueden constituir un mecanismo para prevenir la apoptosis, en un intento para desacoplar la ruta dependiente de p38. Además de su papel en la apoptosis, p38 es necesaria para la inhibición por contacto en un modelo de MEFs p38KO (Faust, Dolado et al. 2005). Por lo tanto creemos que la falta de p38 puede ser responsable en parte de la alta densidad de saturación de estas células en cultivo. Además, varios estudios llevados a cabo por diferentes grupos (incluido el nuestro) han demostrado que la expresión de NQO1 depende de la confluencia del cultivo en células adherentes normales y tumorales. NQO1 se ha encontrada incrementada en cultivos a alta densidad de células BALB/c 3T3 (Schlager, Hoerl et al. 1993), en osteoblastos humanos (Collin, Lomri et al. 2001) y en células HeLa (Bello, Gomez-Diaz et al. 2001, Bello, Gomez-Diaz et al. 2004).

La inyección de MEFs NQO1KO en ratones inmunodeficientes generó el desarrollo de teratomas, mientras que los MEFs silvestres se mostraron inactivos. Cuando examinamos los cortes de estos teratomas mediante microscopía de campo claro, se observaron diversas células y tejidos de origen mesodérmicos (como células de tipo fibroblástico, tejido adiposo pardo y blanco, músculo), destacando el mayor potencial proliferativo y de diferenciación de los MEFs carentes de NQO1.

Los MEFs NQO1KO presentaron además mitocondrias más pequeñas, siendo más abundantes y ocupando una mayor superficie de citoplasma. Alteraciones en los procesos

de fusión y fisión podrían explicar estas diferencias entre los dos genotipos. De hecho, hemos observado como dos proteínas de fusión, Opa1 y Mfn2, estaban disminuidas respecto a los controles, mientras que no hemos observado diferencias en marcadores de fisión. Esto es consistente con un estudio reciente que relaciona NQO1 con el mantenimiento de la integridad mitocondrial (Kwon, Han et al. 2012).

En conjunto, nuestros resultados indican que, aunque Nrf2 y NQO1 participan en el mismo eje de señalización antioxidante (Rushmore, Morton et al. 1991), la deleción de Nrf2 o NQO1 en MEFs genera unos fenotipos celulares muy diferentes.

Introduction

1. Cell Redox State

1.1. General concepts

The cell redox state is the result of the balance between oxidant and reductant molecules in the cell (Halliwell and Gutteridge 1989). This balance plays a role in the survival of the cell since redox state determines the functional status of many molecules and their potential interactions. Any deviation to a more oxidant or more reducing situation can trigger a signaling cascade difficult to predict. The term redox signaling is used in any regulatory process mediated by redox changes in signaling molecules. The metabolism of the cell generates unavoidable modification of the redox condition, either in a compartment or in the whole cell. Also, when interacting with the extracellular medium, the cell has to respond to exogenous prooxidants or antioxidants, readjusting its own balance in order to not compromise the progress of its functions. Changes of the intracellular redox state accompany different cell states such as proliferation, differentiation, senescence or death. It is known that the redox balance conditions gene expression through different pathways which include the modulation of the transcription factors and direct modifications of regulatory proteins as kinases or phosphatases (Droge 2002).

Cell have several components with oxidant or reducing properties. In accordance with Halliwell and Gutteridge (1989) oxidants are chemical species that accept an electron from another species or donate an oxygen. A reducing agent is an element or compound that loses or donates an electron to another chemical species in a redox chemical reaction or accepts an oxygen. Usually the same species present an oxidized form (susceptible of undergoing reduction like $NAD(P)^+$ or FAD) and another reduced form (that can be oxidized like NAD(P)H or $FADH_2$). Antioxidants can be defined as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. The term “antioxidant” is used more often than reducing agent. Sometimes both terms seem to be used as synonyms but they designate different features. Scavenger is frequently used to refer to any substance that is able to eliminate any reactive species. Free radicals and other nonradical species are found in cells and tissues in variable concentrations, being the main cellular oxidant agents.

The reactive oxygen species (ROS) are a group of oxygen-derived molecules, either radical or not, which display high chemical reactivity, whereas and reactive nitrogen species (RNS) are nitrogen-derived molecules. The concentration of reactive species in a cell compartment is determined by the balance of their production (induced or as a product of cell metabolism) and their removal. Composition of the antioxidant defense machinery varies

depending on the tissue and the cell type but, in general terms, we can find the following categories:

a) Agents that catalyze the removal of free radicals and other reactive species like superoxide dismutase enzyme, catalase, peroxidases and thiol-specific antioxidant proteins (Halliwell and Gutteridge 1989).
b) Proteins such as transferrins, hemopexins and methallothioneins that minimize the levels of prooxidants like iron and cupper ions and heme groups. In this group we can also include proteins that oxidize ferrous ion like ceruloplasmin (Halliwell and Gutteridge 1989).
c) Low molecular weight agents that remove ROS and RNS. In this group we can include vitamin C, glutathione, thioredoxin, coenzyme Q and α-tocopherol, some of them being provided from the diet (Halliwell and Gutteridge 1989).
d) Free amino acids could be also considered like ROS scavengers. They are low-efficiency antioxidants but because of its intracellular high concentration they are quantitatively important ROS scavengers (>0.1M) (Droge 2002).
e) Degradative enzymes of oxidized molecules (nucleases, proteases...) (Cadenas and Davies 2000).
f) Proteins that protect biomolecules from oxidative damage. For instance, chaperones (Niforou, Cheimonidou et al. 2014).

In normal conditions, the levels of ROS in cells and tissues are maintained. The imbalance of the cell redox state triggers cellular responses *via* redox signaling. These alterations can be induced by endogenous systems that produce or remove reactive species. These endogenous systems can be in turn regulated by the cell both at the beginning and the end of the signal pathway, and also the intensity of this signal. In other cases the imbalance of the redox state can be triggered by external factors. If variations are not very high, a light adjustment brings the cell to normal conditions. In other circumstances the antioxidant response may be not enough to restore the balance (Figure 1).

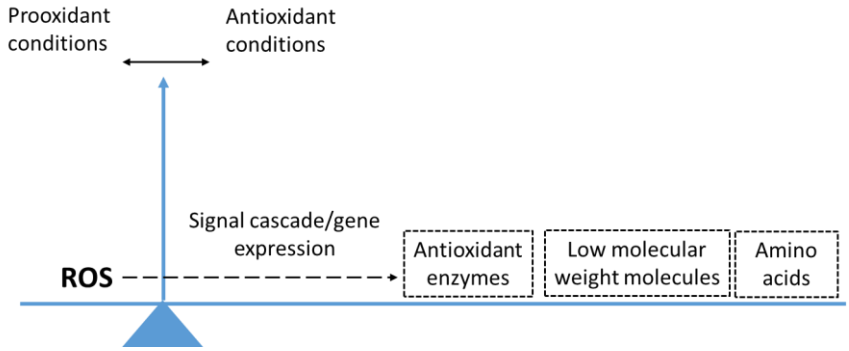

Figure 1: Mechanisms of redox homeostasis. The levels of ROS are determined by the rate of ROS production and their removal by scavenging mechanisms. Certain antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase, catalase, and thioredoxins are potent ROS scavengers. The cellular machinery against ROS also include small-molecule antioxidants, as well as amino acids and proteins. Although the latter substances are less effective than the classical antioxidants on a molar basis, their intracellular concentration is 0.1 M and thus, they may play a prominent role in the overall capacity of cells to counteract ROS. Figure modified from (Droge 2002).

"Oxidative stress" denotes a situation characterized by an imbalance between the reactive species production and antioxidant defenses, independently of their origin. According to Helmut Sies, oxidative stress can be defined as the disturbance in the prooxidant-antioxidant balance, in favor of the former, producing a potential damage (Sies and Cadenas 1985), referred to as "oxidative damage". This situation can be generated by any of these reasons:

a) A decrease in antioxidant enzymes or the reduction of the contribution of antioxidants from the diet.
b) A higher exposition to endogenous or exogenous ROS/RNS. In the former case because of alterations of respiratory mitochondrial chain or NAD(P)H oxidase, whereas in the latter case this can be due to hyperoxia or to environmental contaminants.

In healthy organisms the production of ROS and RNS is basically balanced by antioxidant systems. However, the balance is not perfect and some reactive species causes damage in molecules constantly. These damaged molecules need to be repaired (like the DNA) or removed (the majority of oxidized proteins are removed by the proteasome). Oxidant environments are associated to xenobiotics toxicity, aging and some pathologies.

1.2. Reactive Oxygen Species (ROS)

According to Halliwell and Gutteridge (1989), a free radical is any chemical species capable of independent existence that contains one or more unpaired electrons. The presence of one

or more unpaired electrons causes the species to be attracted slightly to a magnetic field and sometimes makes the species highly reactive. Free radicals can be generated by the loss of one electron from a non-radical or by the gain of one electron by a non-radical. The lower the redox potential of a substance, the better is its electron-donating capacity. The interaction of a non-radical with a radical leads to the generation of a new radical. The term reactive oxygen species is used to refer to the oxygen derivatives, including both radical and non-radical species (Figure 2).

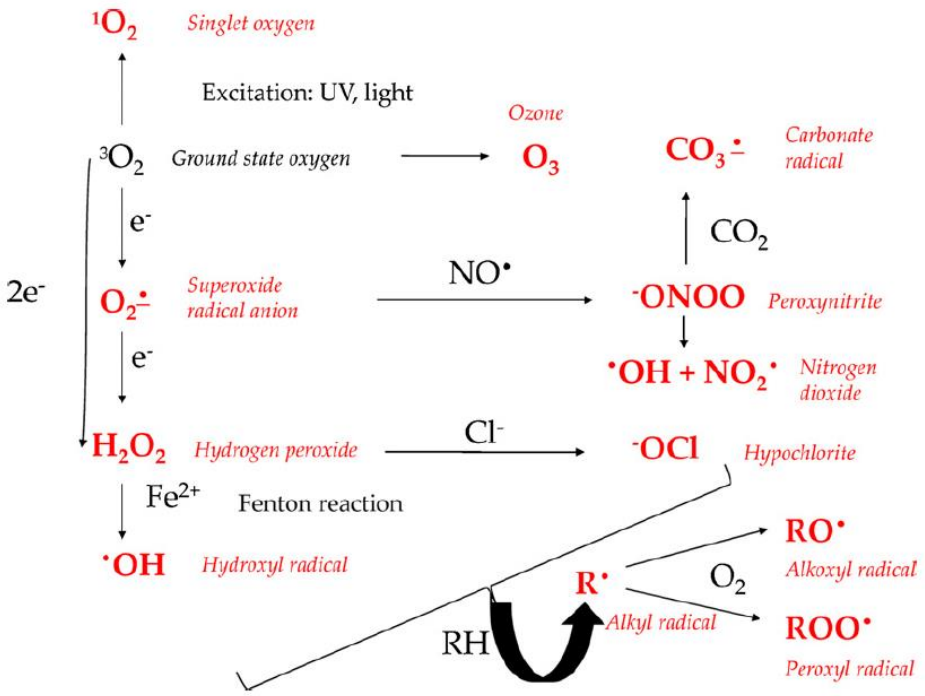

Figure 2: Main Reactive Oxygen species Molecular oxygen is relatively non-reactive but oxygen derivatives are more prone to participate in chemical reactions. Reactive oxygen species (ROS) are formed during aerobic metabolism and in the environment. Figure taken from (Bartosz 2009).

The most important ROS are characterized by the following properties:

a) **Hydroxyl radical (·OH):** This is the most reactive of the ROS and has a very high redox potential (E° OH•, H+/H2O = 2.31 V). Hydroxyl radicals can be generated by the Fenton reaction (Fe^{2+} + H_2O_2 → Intermediate complex → Fe^{3+} + ·OH + ·OH) or by homolytic fission (when a covalent bond is broken and one electron from each of the shared pair remains with each atom) induced by ultraviolet radiation. It can be generated also from ozone in aqueous solution, in the ethanol metabolism and other processes. Hidroxyl radicals rapidly react with biomolecules like nucleotides, lipids and amino acids.

b) **Superoxide Radical ($O_{2}^{\cdot-}$):** This anion radical can be generated when molecular oxygen is reduced by gaining one single electron. Some enzymes produce $O_{2}^{\cdot-}$ and the most important one is xanthine oxidase, even though is not a common *in vivo* process. Several biologically-important molecules auto-oxidize in the presence of O_2 to yield $O_{2}^{\cdot-}$. These include glyceraldehyde, $FMNH_2$, $FADH_2$, adrenaline, dopamine and thiol compounds such as cysteine. It has been suggested that these auto-oxidation reactions need for the presence of transition metals like iron or copper. Superoxide radicals (*E*º O2·-, 2H+/H2O2 = 0.94 V) are less reactive in aqueous solutions in comparison with ·OH. However, superoxide can generate an important damage to hydrophobic membranes. The protonated form of $O_{2}^{\cdot-}$, hydroperoxyl radical ($HO_{2}^{\cdot}$) is more reactive than the superoxide anion and can initiate peroxidation of fatty acids. In addition to its direct effects, superoxide can be cytotoxic because of the generation of other reactive species. Superoxide rapidly react with NO· to yield peroxinitrite (ONOO-) which, in turn, produces more noxious species under physiological conditions. Also, the combination of superoxide and hydrogen peroxide in the presence of catalytic metal ions generates hydroxyl radical by the Haber-Weiss reaction: $H_2O_2 + O_{2}^{\cdot-} \rightarrow O_2 + {}^{\cdot}OH + {}^{-}OH$.

c) **Hydrogen peroxide (H_2O_2):** It is the more biologically-important non-radical oxygen species. Hydrogen peroxide can cross cell membranes and cause damage like the inactivation of glyceraldehyde 3-phosphate dehydrogenase. At high concentrations (10-100 μM) is cytotoxic. The most toxic characteristic of hydrogen peroxide is its capability of easily reacting with metal ions to produce ·OH by Fenton chemistry. As stated above, it can also react with superoxide to generate ·OH by the Haber-Weiss reaction.

1.3. The two faces of ROS

ROS are not only produced from exogenous sources (ultraviolet radiations, pollutants, phenolic compounds, smokes…), but also the normal functioning of the cell is a source of ROS. Cells can generate ROS as byproducts of metabolism or in an active way. In the case of neutrophils and macrophages there is a production of $O_{2}^{\cdot-}$ in chronic and acute inflammation states. The uptake of oxygen is linked to the activation of a NADPH-oxidase, a master key in the defense against infections and tumor cells. In peroxisomes H_2O_2 is produced as a byproduct of the long chain fatty acid oxidative metabolism. The activities of lipoxygenases, cyclooxygenases, cytochrome P450 and myeloperoxidases are also sources of ROS (Halliwell and Gutteridge 1989).

Mitochondria are considered as the main cellular source of superoxide formed by one-electron reduction of oxygen, a side reaction of the respiratory chain. The probability that molecular oxygen is reduced to superoxide rather than to water depends on the proton concentration gradient. The probability of generating superoxide is increased if the proton concentration gradient across the mitochondrial inner membrane is high and the proper flux of electrons through the respiratory chain is less favored (and even more if the ADP availability is low). Superoxide release from mitochondria depends dramatically on the proton motive force. This can be explained because under high proton-motive force situations protons are not easily pumped out of the matrix against the electrochemical gradient. As a result, the activity of the electron-transport chain slows down, the half-lives of reduced intermediates are longer and thus, the chance for their one-electron reaction with oxygen becomes higher (reviewed by (Bartosz 2009)).

It is well established that ROS inflict oxidative damage that may cause DNA mutations, protein inactivation and cell death. An important role for ROS in several diseases and biological processes such as aging, some types of cancer, atherosclerosis, neurodegenerative diseases and chronic inflammatory situations has been also demonstrated (Droge 2002). However, there is abundant data demonstrating that ROS have important roles in cell signaling since they can be participant or modifiers of signaling pathways such as those involved in the regulation of proliferation. The persistence of ROS presence in cells indicates an evolutionary selection to perform roles in cellular metabolism (Barja 1993). A physiological rate of ROS production activates cellular signaling pathways that are necessary for cell growth and proliferation, while an excessive production of ROS, overmatching the antioxidant capacities of the cell, leads to oxidative stress that results in metabolic disturbances and cell death. In normal cells, basal levels of H_2O_2 are low and their increase is first associated with cell growth promotion. In tumor cells, high levels of H_2O_2 are associated with their rapid cell growth phenotype. A further increase in intracellular H_2O_2 inhibits tumor cell proliferation (Nicco, Laurent et al. 2005).

In general terms a signaling molecule has to fulfill the following requirements: 1) A tight control of its concentration, at the levels of both synthesis and removal; 2) the existence of specific receptors and 3) the reversibility of the signaling effect. These consideration point to hydrogen peroxide and/or superoxide as the best candidates for signaling ROS. However, it is not always easy to distinguish between the effects of these two ROS because superoxide has a short life and is rapidly transformed into hydrogen peroxide (Bartosz 2009).

According to Bartosz (2009), the ways by which ROS act as signaling messengers fall into these two (not mutually exclusive) mechanisms:

a) **Modification of target molecules:** Sometimes ROS may irreversibly damage biomolecules, as is the case for hydroxyl radicals. However, superoxide and hydrogen peroxide are mild oxidants. If transition metals are not present (thus avoiding Fenton reaction), H_2O_2 has as main target the thiol group of cysteine residues, inducing the formation of disulfides. The most susceptible of undergoing thiol oxidation are phosphatases, G proteins, a number of ion channels and some transcription factors. Since the level of substrate phosphorylation relies on the balance of phosphorylation and dephosphorylation rates, the inactivation of phosphatases by ROS increases the phosphorylation level of many proteins. In addition, it has been suggested that some kinases can be directly activated by ROS.
b) **Changes in the redox state of the cell:** This can be defined by the values of redox couples like Glutathione disulfide/Glutathione (GSSG/GSH), NAD^+/NADH and $NADP^+$/NADPH, among others. ROS action can be mediated by changes in the redox state of the cell and alterations of this parameter can be the trigger for several signaling processes.

2. Redox state and the cell cycle

2.1. The cell cycle

The cell cycle is divided into four phases consisting in: DNA synthesis (S phase), cell division (M phase) and two gap periods between S and M phase: G1 phase (gap1) located between M and S phases, and G2 (gap2) located between S and M. Unlike the rapid proliferation that characterizes embryonic cells, some adult cells completely stop their division or only divide occasionally. Cell cycle progression depends on the integration of a number of extra- and intra-cellular signals and intrinsic checkpoints. The G1/S and the G2/M checkpoints ensure that damaged or incomplete DNA is not passed on to daughter cells. Cell cycle progression can be stopped at these points in order to repair the DNA damage. The so-called G1 restriction point is regulated by external factors like growth and adhesion factors. In the presence of growth factors, cells can surpass the restriction point and enter the S phase. Once a cell surpasses this point, it is committed to progress through the S phase and the rest of the cell cycle, even with the absence of growth factors. On the contrary, if the adequate factors are not present during the G1 phase, progression of cells is halted at the G1 restriction point and cells thus enter quiescence (G0). Cells in G0 have a lower metabolic rate than cells in G1 and they stay at this state until the right signals are perceived to induce

their continuation through the cell cycle. Other checkpoints located in mitosis ensure proper progression through this phase. For instance, the Sp checkpoint controls that the chromosomes are bound correctly to the mitotic spindle.

The cell-cycle control system is based on two key families of proteins: Cyclins and cyclin-dependent protein kinases (CDKs). CDKs induce processes by phosphorylating selected proteins on serine and threonine residues. Cyclins bind to CDKs and control their kinase activity and their specificity towards protein substrates. Cyclin-CDK complexes assembly, activation and disassembly are the pivotal events driving the cell cycle. Cyclins are so called because they undergo a cycle of synthesis and degradation with every cell division cycle. Each cyclin interacts with a specific CDK. The most important cyclin-CDK complexes in mammals are formed by CDK4/6 (with cyclins D) and CDK2 (with cyclins E and A) in the G1 phase and in G1/S interphase, and by CDK1 (with the cyclins A and B) during mitosis. The complex formed by cyclins D and CDK4 or CDK6 (depending on the cell type) is the first complex that is activated during G1. As G1 progresses the cyclin E production is increased and. at the end of G1 it reaches its highest level. The association of cyclin E and CDK2 determine the surpassing of the G1 restriction point while the complexes formed by cyclin A and CDK2 are responsible of the S phase initiation. In the S phase cyclin E is degraded (Sherr 1993). Next, cyclin A and B interact with CDK1 and promote the entry in G2/M. The cyclin A/CDK1 complexes reach their highest activity during the G2 phase but then they are degraded immediately to allow that the cyclin B/CDK1 complexes induce the entry into mitosis. Proteolytic removal of cyclin B determines the end of the mitosis (Peeper, Parker et al. 1993). A regulatory strategy is that the same protein initiates one phase of the cycle and inhibits another one. For instance, the cyclin-CDK complexes needed for the G1/S transition inhibit the G2/M transition. In this way, the cell cycle progression is irreversible (Figure 3). Once the respective regulation point is surpassed, cyclins undergo ubiquitylation-mediated proteolysis (Hershko 1997).

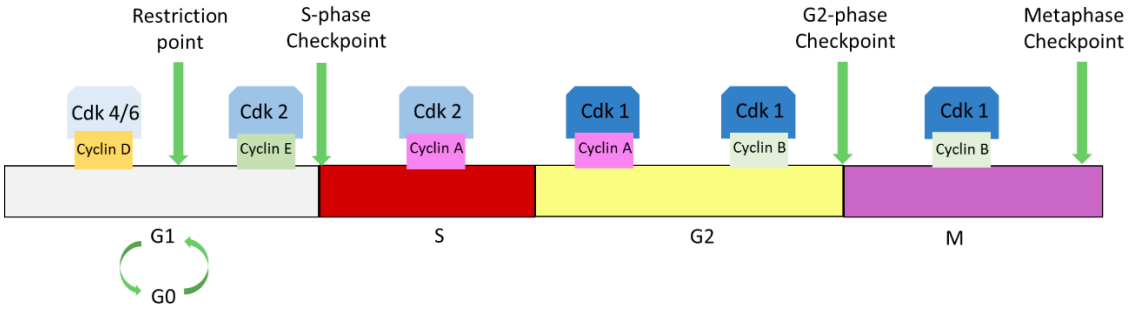

Figure 3: Cell cycle regulation in mammals. Transition between different phases (G1, S, G2 and M) is regulated by the formation of cyclin-Cdk complexes. G0 is a quiescent state of the cells. The exit from G0 can be determined by mitogenic signaling.

CDKs are not only regulated by association/disassociation with cyclins. Its activation also requires a threonine residue phosphorylation catalyzed by CDK-activating kinase (CAK), a Cdk7/cyclin H complex (Kaldis 1999). The cell cycle progression depends as well of the activity of the cyclin-kinase inhibitors (CKI). They are divided into two families based on the structures and affinities: INK4 and Cip/Kip (CDK inhibitory proteins/kinase inhibitory protein). INK4 family consists of four members (p16, p15, p18 and p19). They bind and inhibit specifically CDK4/6. Cip/Kip family is formed by p21, p27 and p57 and they have a wider affinity range in the CDK inhibition (reviewed by (Vidal and Koff 2000)).

In mammal cells, one of the most important substrates of the cyclin/CDK complexes in phase G1 is the retinoblastoma protein (pRB), a tumor suppressor. Activity of pRB is determined by its phosphorylation grade that depends on the cell cycle phase. When hypophosphorylated, pRB is bound to several transcription factors, especially E2F family members. This family is formed by five different isoforms that form heterodimers with a second group of proteins called DP1. pRB is hypophosphorylated at the beginning of G1 whereas its phosphorylation degree increases as the G1 phase progresses. The phosphorylation triggers the release and activation of the E2F transcription factors, allowing the transcription of genes involved in the phase S initiation, like cyclin E. Phosphorylaton of pRb is carried out initially by the cyclinD-CDK4/6 complexes. The cyclin E/CDK2 complexes complete the phosphorylation of pRb at the end of G1 and determine the beginning of the S phase (Herwig and Strauss 1997).

P53 is also very important in the cell cycle regulation. In normal conditions p53 levels are low because of its short life. However, intra and extracellular stress induce stabilization and p53 activation. It induces the transcription of different genes whose expression condition the progression of the cell cycle (for instance, p21 is regulated by p53). The activity of p53

is particularly increased after DNA damage, inducing the cell cycle detention which allows for the subsequent DNA reparation (Levine 1997).

The cell cycle progression in mammals is determined by a number of extracellular signaling molecules, like growth factors or interaction with extracellular matrix components. The extracellular signaling acts mainly in cells that are on G1 phase through G-proteins. The activation of the receptors triggers a signaling translation cascade that induce cell proliferation (Assoian and Schwartz 2001, Boonstra and Moes 2005).

2.2. Alteration of the redox balance in the cell cycle

Cell cycle progression is regulated by external factors, mainly nutrients and growth factors. Growth factors act through a complex intracellular network and eventually induce the cell cycle progression or detention. There are many cell cycle regulators and, among them, we can also find factors that condition the cell redox state.

The first reference to a link between cell cycle and redox state of the cell was published in 1931, when Rapkine showed that the cell thiols of the sea urchin eggs fluctuate cyclically (reviewed by (Mauro, Grasso et al. 1969)). Mauro and collaborators in 1969 studied how the levels of sulfhydryl (-SH) and disulfide (-SS-) groups change along the different phases of the cell cycle in HeLa cells. Levels of non-protein –SH groups decrease 10 times along the G1 phase, but rise abruptly as the cell enters in S phase to increase 30 times at the end of the replication phase. However, the protein –SH groups increase during G1 to fall during S phase (Mauro, Grasso et al. 1969).

Almost two decades ago changes in redox potential (E) were measured from the concentrations of GSH and GSSG using the Nernst equation,

$$E = E_0 + k\log([GSSG] / [GSH]^2)$$

Where [GSSG] and [GSH] are, respectively, concentrations of oxidized and reduced form of glutathione. E_0 and k are constants. These two modulators of the cellular redox state were measured in cultures of proliferating cells at varying densities, which exhibited contact inhibition of proliferation. Redox potentials of normal cells were more reducing in proliferating (-220 mV) than in confluent cultures (-188 mV). The treatment with buthionine sulfoximine, an inhibitor of GSH synthesis leading to GSH depletion, induced a less reducing redox state and decreased proliferation (Hutter, Till et al. 1997). It has been reported that GSH levels are significantly higher in G2/M than G1 phase. This suggest that G2/M cells have a higher reducing state than G1, with S phase cells having intermediate

levels (Conour, Graham et al. 2004). On the other hand, the intracellular distribution of GSH is also known to change with growth in cultures of 3T3 cells. GSH, which is located mainly in the nucleus of proliferating cells, is redistributed between nucleus and cytoplasm when cells reach confluence (Markovic, Borras et al. 2007). N-acetyl cysteine (NAC) addition, normally used as thiol antioxidant, inhibits proliferation in Murine Embryonic Fibroblasts (MEFs), hepatic stellate cells and smooth muscle cells (Kim, Rhim et al. 2001, Menon, Sarsour et al. 2003, Kyaw, Yoshizumi et al. 2004). It was thought that the inhibition of cell growth by NAC was due to its reducing action. However, a later study pointed out to the production of superoxide and the subsequent decrease of cyclin D1 levels as the main reasons for this inhibitory effect (Menon, Sarsour et al. 2007). Contact inhibition in fibroblasts is apparently due, at least partially, to a decrease in the intracellular ROS. In fact, when sparse cultures are treated with the reducing agent dithiothreitol (DTT), proliferation is stopped but when H_2O_2 is added to confluent cultures, growth is stimulated despite the contact among cells (Pani, Colavitti et al. 2000). Identical results have been shown for neural precursor cells, which also display higher ROS levels when cultured at low density (Limoli, Rola et al. 2004).

Taking together, all these previous data confirm a tight relationship between the redox state and the cell cycle. In fact, a **Redox Model of Cell Proliferation (RMCP)** has been proposed. The RMCP emphasizes a role for changes in the intracellular redox potential during the cell cycle in helping to regulate normal cell proliferation (Hoffman, Spetner et al. 2008).

The relationship between redox state and cell growth is not exclusive of mammalian cells. Fission yeasts also have a metabolic redox cycle with a fermentative reducing phase and an oxidant respiratory phase. Many of the genes that control DNA replication and cell cycle progression are expressed during the reducing phase (Klevecz, Bolen et al. 2004, Tu, Kudlicki et al. 2005). This is consistent with the results obtained in mammals, showing an increase the reducing state during S and G2/M (see above). *Dictyostelium discoideum* is a soil-living amoeba. Under unfavorable conditions it generates $O_{2^{\cdot-}}$ and makes cell aggregated (Bloomfield and Pears 2003). The use of superoxide as messenger in one of the oldest eukaryotes suggests that redox signaling appeared very early in the evolution scale, being essential for the growth and development of all organisms.

In summary, a number of studies show that proliferating cells have a more reducing state and non-dividing or differentiated cells have a less reducing state. Differences in redox state between cell cycle phases are also observed, being more pro-oxidant in G1 and more reducing in G2/M (Figure 4). Therefore, there exist a tight link between processes that

regulate redox state and processes that regulate the cell cycle, a redox cycle within the cell cycle (Menon and Goswami 2007).

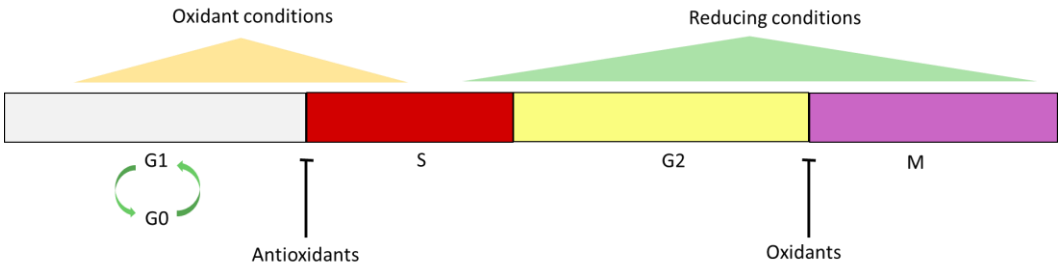

Figure 4: A redox cycle within the cell cycle. Periodic oscillations in intracellular redox state have been described and seem to play a central role in regulating progression from G0/G1 to S to G2 and M cell cycle phases. G2/M cells have a higher reducing state than G1, with S phase cells having intermediate levels.

3. Antioxidant defense mechanisms

3.1. Enzimatic antioxidant defense

3.1.1. Superoxide dismutase (SOD)

SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2. The hydrogen peroxide so generated is then removed either by catalase or by enzymes of the glutathione peroxidase family using GSH.

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Three human SOD isoforms are known: CuZnSOD (SOD1), MnSOD (SOD2) and ECSOD (SOD3). The discovery of SOD in aerobic cells led directly to the proposal that superoxide radicals are the major factor in oxygen toxicity and that the SOD enzymes are an essential defense against them, which constitutes "the superoxide theory of oxygen toxicity" (Fridovich, 1995). However, the existence of anaerobes with SOD and aerobes without SOD also argues against this theory (Halliwell and Gutteridge, 1999).

The MnSOD (SOD2) isoform is located in the mitochondrial matrix. The presence of Mn in the active site is essential for the functioning of the enzyme. In one study where four cell lines showing differential responses to density inhibition were studied it was found that the two cell lines that showed density limitation of growth also demonstrated induction of MnSOD at the time when the cells stopped proliferating in culture, whereas the other two cell lines (with no density limitation of growth) did not show induction of MnSOD. In NIH-3T3 fibroblasts there is a decrease of the levels of MnSOD activity in S in comparison to G_0, which is not observed for other enzymes like catalase, glutathione peroxidase or the

CuZnSOD isoform. These findings suggest that the increase of MnSOD might correlate with decreased cell proliferation. (Oberley, Schultz et al. 1995). Experiments carried out in MEFs have demonstrated that the decrease of MnSOD activity (and the subsequent increase in ROS levels) induce cyclin D1 and B1 expression and cell growth. On the contrary, an increase of the MnSOD activity causes cyclin B1 levels to decrease and facilitates the switch to a quiescent state (Sarsour, Venkataraman et al. 2008). In accordance with these authors, these results confirm the hypothesis that the MnSOD activity regulates a mitochondrial 'ROS-switch' favoring a superoxide-signaling regulating proliferation and a hydrogen peroxide-signaling supporting quiescence. Since mitochondria is the main source of cellular ROS and MnSOD is an antioxidant enzyme located in the mitochondrial matrix, it is possible that the mitochondrial oxidants function as part of the cell cycle signaling machinery (Sarsour, Kalen et al. 2014). It is very interesting that the overexpression of MnSOD inhibits the growth in pancreatic and prostate tumor cells (Ough, Lewis et al. 2004, Venkataraman, Jiang et al. 2005). The complete absence of MnSOD in mice causes death in the first 24 days of life by neurodegeneration. These mice show also four times more DNA damage than normal mice as well as a higher sensibility to hyperoxia (Lebovitz, Zhang et al. 1996).

Almost all eukaryotic cells have the CuZnSOD (SOD1) isoform. In animal cells SOD1 is mainly located in the cytosol but it can be also found in lysosomes, peroxisomes and intermembrane mitochondrial space (Okado-Matsumoto and Fridovich 2001). The latter location is considered very important because of the vicinity of the mitochondrial electron transport chain and the possibility of superoxide being released to this compartment (Muller, Liu et al. 2004). CuZnSOD needs to be activated by oxidative modification of its critical thiol groups (Inarrea, Moini et al. 2005). *Sod1*-/- mice have high levels of oxidative stress and aging-related pathologies. Also, lipid peroxidation levels are two- or three-fold higher in *Sod1*-/- compared with normal mice (Muller, Lustgarten et al. 2007).

The ECSOD (SOD3) isoform of SOD isless abundant and can be found in the extracellular space (in serum and lung). *Sod3*-/- mice are viable, fertile and have a similar lifespan. However they are more sensitive to hyperoxia (Sentman, Granstrom et al. 2006).

3.1.2. Catalase

Superoxide dismutation generates hydrogen peroxide. Catalases and peroxidases are the enzymes that carry out the removal of H_2O_2 in the cell, generating water and molecular oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase can be found in all animal tissues although its activity varies among cell types, the maximal level being found in liver. Catalase can be located in peroxisomes, where the majority of the enzymes that generate H_2O_2 are found (such as glycolate oxidase, urate oxidase or the flavoprotein deshydrogenases involved in the β-oxidation of fatty acids) (Halliwell and Gutteridge 1989). As an exception, catalase was also found in the mitochondrial matrix of rat (Radi, Turrens et al. 1991). Overexpression of catalase inhibits proliferation of vascular smooth muscle cells and induces apoptosis. This indicates that H_2O_2 participates in cell proliferation but also survival (Brown, Miller et al. 1999). Similarly to other antioxidant enzymes, catalase levels are related with cell culture density. Catalase expression is higher in confluent hepatocytes and HeLa cells than in the corresponding low density cultures (Rohrdanz and Kahl 1998, Bello, Gomez-Diaz et al. 2001).

Cat-/- knockout mice have a normal development and are able to remove H_2O_2 despite the lack of catalase. In fact, these mice are not more sensitive to oxidative damage in liver, lung and crystalline. However, differences in mitochondrial function were detected after cortical lesion (Ho, Xiong et al. 2004).

3.1.3. Glutathione peroxidase

The glutathione peroxidase family (Gpx) is a group of enzymes which remove H_2O_2 by reducing it to H_2O and oxidizing GSH:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

These enzymes possess four protein subunits. Each subunit have a selenium atom in the active site in form of selenocysteine. Gpxs are distributed in all animal tissues and specifically use GSH as an electron donor. If they act on fatty acid hydroperoxides and synthetic hydroperoxides, the peroxide is reduced to an alcohol.

$$LOOH + 2GSH \rightarrow GSSG + H_2O + LOH$$

In normal cells the GSH/GSSG ratio is high, which indicates the existence of efficient mechanisms by which GSSG is reduced back to GSH. This conversion is carried out by glutathione reductase:

$$GSSG + NAD(P)H + H^+ \rightarrow 2GSH + NADP^+$$

The glutathione peroxidase 1 (Gpx1) is abundant is all mammalian tissues. Its complete absence in *Gpx1*-/- mice do not produce changes in phenotype. The only feature observed

in these knockout mice is that they develop cataracts earlier than normal mice. Also, *Gpx1*-/- mice are more sensitive to paraquat and diquat but not to hyperoxia (reviewed by (Muller, Lustgarten et al. 2007)). Overexpression of phospholipid hydroperoxide glutathione peroxidase (PhGpx) inhibits the transition from G1 to S in the cell cycle. This suggest the implication of the lipid peroxidation in the cell cycle (Wang, Schafer et al. 2003).

Even though more attention has been focused on the main antioxidant enzymes (SOD, catalase and Gpx), there is also information about other enzymes that collaborate in the maintenance of the redox balance, demonstrating the complexity and importance of this system.

3.1.4. Other antioxidant enzymes

Other antioxidant defenses include the **thioredoxin system** that is constituted by thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. This system plays an essential role in the redox maintenance of the thiol groups in proteins (Arner and Holmgren 2006). Thioredoxin is a 12 KDa polypeptide that can be found in prokaryotes and eukaryotes. In mammals it is widely distributed, being more concentrated in the endoplasmic reticulum and to a lesser extent in the cell surface (Halliwell and Gutteridge 1989). Thioredoxin possess two cysteines in the active site. The two reduced sulfhydryl groups are converted into a disulfide group in the oxidized enzyme:

$$Trx\text{-}(SH)_2 + protein\text{-}S_2 \rightarrow Trx\text{-}S_2 + protein\text{-}(SH)_2$$

Thioredoxin reductases of mammals are flavoprotiens with active site containing a selenocysteine that reduce thioredoxin:

$$Trx\text{-}S_2 + NADPH + H^+ \rightarrow Trx\text{-}(SH)_2 + NADP^+$$

High levels of thioredoxin and its reductase are found in several types of human cancer (Arner and Holmgren 2006, Urig and Becker 2006). The ablation of Trx2 (only localized in the mitochondria) in B cells of chicken produces ROS increase, release of cytochrome *c* to the cytoplasm and apoptosis (Tanaka, Hosoi et al. 2002). In fact, *Trx2*-/- mice die during the embryonic development by massive apoptosis (Nonn, Williams et al. 2003). Similarly, genetic deletion of the isoform 1 or 2 of thioredoxin reductase is lethal in mice (Conrad, Jakupoglu et al. 2004, Jakupoglu, Przemeck et al. 2005). Several studies have linked the

thioredoxin system to the cell cycle. Reduced thioredoxin can promote proliferation, inhibit apoptosis and protect the cells against oxidative stress (Powis and Montfort 2001).

Peroxiredoxins (Prxs) are part of the antioxidant machinery of the cell. The majority of these enzymes display thioredoxin peroxidase activity. Their substrate are H_2O_2, organic peroxides and peroxynitrite. All Prxs share the same basic catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by the peroxide substrate. There are six peroxiredoxins (Wood, Schroder et al. 2003). Prx1 is abundant in erythrocytes but it can be also found in the majority of the tissues. Knockout mice for *Prx1* gene have hemolytic anemia and increased incidence of cancer as well as more oxidative damage in DNA and proteins. Prx2 has the same distribution as Prx1. *Prx2*-/- mice also display hemolytic anemia and age earlier than normal mice. *Prx6*-/- are more sensitive to hyperoxia and paraquat. Mice lacking catalase and glutathione peroxidase do not develop similar pathologies as those lacking Prx1 or Prx2, suggesting that peroxiredoxins are critical enzymes in removing H_2O_2. The possibility exists that cellular location and abundance may explain why the loss of peroxiredoxins is more deleterious than the loss of potentially more important enzymes (Wood, Schroder et al. 2003, Muller, Lustgarten et al. 2007). Prx activity can be regulated by phosphorylation. Phosphorylation of Prx1 at Thr 90 results in an over 80% activity decrease. Several cyclin-dependent kinases (Cdk2, Cdk4 and Cdk6) are able to phosphorylate this enzyme; and such phosphorylation occurs during mitosis. Prx2 can be phosphorylated by Cdk5, which similarly decreases its activity (Bartosz 2009).

NADH-cytochrome *b*$_5$ reductase (Cytb5R) is an integral membrane protein mainly located in endoplasmic reticulum and outer mitochondrial membrane, although a small fraction can be found in the plasma membrane. Soluble forms have also been described in erythrocytes. Its functions are fatty acid desaturation and elongation, cholesterol biosynthesis, drug metabolism and methemoglobin reduction in erythrocytes (Halliwell and Gutteridge 1989). Another Cytb5R function is the regeneration of hydrophilic and lipophilic antioxidants as ascorbate and vitamin E (Constantinescu, Maguire et al. 1994, Shirabe, Landi et al. 1995). Cytb5R in the plasma membrane can also act as a quinone reductase. It reduces ubiquinone *in situ* and in reconstituted liposomes (Villalba, Navarro et al. 1997). Our group have demonstrated that Cytb5R levels are significantly higher when HeLa cells are confluent than in low density cultures (Bello, Alcain et al. 2003). This is consistent with another study carried out in HepG2 cells (hepatoma cells) (Doostdar, Demoz et al. 1990).

Methionine sulfoxide reductase A (MsrA) catalyzes the reduction the reduction of methionine sulfoxide to free or protein-bound methionine. This widely

expressed enzyme constitutes an important repair mechanism for oxidatively-damaged proteins, which accumulate during the manifestation of certain degenerative diseases and in aging processes. Different isoforms have been located in cytoplasm, nucleus, mitochondria and endoplasmic reticulum (Hansel, Kuschel et al. 2002, Kim and Gladyshev 2004). Overexpression of MsrA increases lifespan in flies and yeasts. In mice, the lack of MsrA shortens lifespan in a 40% and increases sensitivity to hyperoxia (reviewed by (Muller, Lustgarten et al. 2007)).

3.2. Non-enzimatic antioxidant defense

3.2.1. Metal-binding proteins

A number of proteins such as transferrin, ferritin, lactoferrin, ceruloplasmin and albumin bind transition metal ions. These proteins can be considered as non-enzymatic antioxidants that act by sequestrating transition metal ions responsible for generating the most reactive oxygenated radical species, as hydroxyl radicals (Sung, Hsu et al. 2013). Ferritin is an intracellular protein that prevents the accumulation of iron. Ceruloplasmin is present in the plasma, where it removes ferrous ion and also binds copper ions, which are sequestered and their deleterious effects reduced (Solomons 1979). Albumin, the most abundant protein in the plasma, also constitutes the major source of extracellular reduced sulfhydryl groups, which act as potent scavengers of ROS (Bernardi, Ricci et al. 2014). Albumin also possesses the capacity of binding copper tightly and iron weakly, thus avoiding hidroxyl radical formation (Sitar, Aydin et al. 2013).

3.2.2. Low molecular weight agents

Some exogenous and endogenous non-enzimatic antioxidants such as the water-soluble vitamin C, glutathione, uric acid and the lipid-soluble substances coenzyme Q and α-tocopherol also exert their protective role against oxidative stress.

The role of GSH as a substrate for the H_2O_2-removing enzyme has already been discused. A number of studies emphasize the importance of the GSH/GSSG redox pair in maintaining the cell redox state. In fact, the GSH/GSSG system is considered as the main cell redox buffer. The average concentration of GSH in the cytosol is 1-11mM, far above any other redox substance in the cell. For instance, it is estimated that the thioredoxin concentration is 100 to 1000-fold lower than glutathione (Jezek and Hlavata 2005). For this reason the quantification of the GSH and GSSG levels are frequently used to determine the redox state of the cell, matching the GSH/GSSG ratio to the redox state (Schafer and Buettner 2001, Hoffman, Spetner et al. 2008). Finally, it is important to underline the role of the cysteine/cystine in maintaining the extracellular redox balance in mammal plasma. It has

been proven that alteration of this redox pair condition the communication between cells and tissues (reviewed by (Go and Jones 2008)).

Regarding the reduced form of coenzyme Q (ubiquinol), it is known that this electron-carrier of the mitochondrial electron transport chain also play a role in cellular membranes and plasma lipoproteins protecting these structures against radical damage. In addition to its capacity to trap lipid peroxyl radicals, ubiquinol has demonstrated to be an efficient regenerator of α-tocopherol (reviewed by (Pisoschi and Pop 2015)).

Exogenous antioxidants like ascorbic acid (vitamin C) or vitamin E also play an important role in the antioxidant defense. The concentration of ascorbate in plasma from healthy humans ranges between 40 and 80 μM. At these levels ascorbate functions as an endogenous antioxidant. It serves as a co-antioxidant with vitamin E to protect low density lipoproteins (LDL) from oxidative damage induced by peroxyl radicals. Ascorbate is able to donate one electron to a number of potentially damaging oxidizing radicals such as hydroxyl, alkoxyl or peroxyl radicals. This one-electron oxidation of ascobate results in the production of the ascorbyl radical which is relatively unreactive, and can be reduced back to ascorbate by NADH- and NADPH-dependent reductases (reviewed by (Du, Cullen et al. 2012)). An important feature of the action of ascorbate is its synergistic interaction with vitamin E, a primary antioxidant in LDL and lipid membranes. In this context, ascorbate as well as other antioxidants such as ubiquinol and polyphenolic compounds are important for maintaining vitamin E and inhibiting oxidation of lipids (Du, Cullen et al. 2012, Niki 2014).

Uric acid, a compound that accumulates as an end-product from purine metabolism, also exerts its function as an antioxidant. As an antioxidant molecule, uric acid is mainly a scavenger of singlet O_2 and peroxyl and hidroxyl radicals, but also of ozone and hypochlorous acid (Halliwell and Gutteridge 1989).

4. Nuclear factor E2-related factor-2 (Nrf2)

To neutralize oxidative stress insults, cells have acquire a complex mechanism of defense in which induction is highly coordinated. This coordinated response is regulated through a *cis*-regulatory element called the antioxidant responsive element (ARE) or electrophile-responsive element (EpRE) within the regulatory region of target genes (Friling, Bensimon et al. 1990, Rushmore, Morton et al. 1991). Nrf2 (Nuclear factor erythroid 2 related factor, NF-E2-related factor 2) (Moi, Chan et al. 1994) has been identified as the major regulator of ARE-mediated gene expression (Venugopal and Jaiswal 1996, Itoh, Chiba et al. 1997, Ishii, Itoh et al. 2000). The ARE/EpRE core sequence (TGACXXXGC) has been reported as the consensus sequence of the DNA binding element for Nrf2 gene regulation (Rushmore, Morton et al. 1991, Itoh, Chiba et al. 1997). The ARE-directed genes are involved in a variety of protective actions including direct inactivation of ROS (e.g. catalase, SOD) (Hur and Gray 2011), GSH *de novo* generation and homeostasis regulation (Harvey, Thimmulappa et al. 2009), Trx (Im, Lee et al. 2012) and TrxR induction (Reddy, Kleeberger et al. 2007) and many others antioxidant-defense genes.

4.1. The CNC transcription factor family

In many organisms the cytoprotective response to manage oxidative and electrophilic challenges is controlled, in large part, by the Cap N' Collar (CNC) transcription factors, which constitute a unique subset within the bZIP (basic-leucine zipper domain) transcription factor family (which share a highly conserved basic region-leucine zipper) (Mathers, Fraser et al. 2004). In mammals, the CNC transcription factors include p45-NFE2, Nrf1, Nrf2, Nrf3, as well as two other distant members, Bach1 and Bach2 (Motohashi, O'Connor et al. 2002). Phenotypic analysis of mice containing targeted deletions of individual or multiple CNC family members has revealed that Nrf1 and Nrf2 are the major regulators of cytoprotective gene expression (Leung, Kwong et al. 2003). Nrf1 and Nrf2 share a common domain structure, including a conserved N-terminal regulatory domain termed Neh2, a central transactivation domain, and a C-terminal bZIP domain required for heterodimer formation with members of the small Maf protein family, for DNA binding and nuclear import and export (Moi, Chan et al. 1994, Chan, Lu et al. 1996, Katoh, Itoh et al. 2001, Bloom, Dhakshinamoorthy et al. 2002, Motohashi, O'Connor et al. 2002, Komatsu, Kurokawa et al. 2010, Mitsuishi, Motohashi et al. 2012). However is Nrf2 (Moi, Chan et al. 1994) the major regulator of ARE-mediated gene expression (Venugopal and Jaiswal 1996, Itoh, Chiba et al. 1997, Ishii, Itoh et al. 2000).

4.2. Nrf2 structure

Several homologue domains were identified when Nrf2 genes from different species, such as human, mouse, and chicken, were aligned (reviewed by (Zhang 2006)). The domains are identified as Neh 1–6 (Figure 5). The N-terminal domain is Neh2. Neh2 domain binds to the Kelch domain of the Nrf2 negative regulator Kelch-like ECH-associated protein 1 (Keap1) (Itoh, Wakabayashi et al. 1999). The Kelch domain of Keap1 binds to two binding sites located in Neh2: a high-affinity ETGE motif and a low-affinity DLG motif. (Tong, Katoh et al. 2006, Tong, Padmanabhan et al. 2007). Neh2 domain contains seven lysine residues in the α-helical region for ubiquitin conjugation that lead to a negative regulation of the Nrf2 activity through proteasome-mediated degradation (Zhang, Lo et al. 2004). The Neh4 and Neh5 are indispensable in order to achieve maximum transactivation activity. They are two acidic residues-rich transactivation domains that interact with CREB-binding protein (CBP) both independently and cooperatively (Katoh, Itoh et al. 2001). Regarding Neh6, it has a serine-rich conserved region although its function remains largely unknown. In addition, it possesses a nuclear localization signal (NLS) which is required for Nrf2 nuclear import. Deletion of the NLS causes the loss of nuclear localization of Nrf2 and diminishes transcriptional activity of downstream genes (Jain, Bloom et al. 2005). The Neh1 domain contains a CNC homology region and bZIP domain, which is necessary for DNA binding and heterodimer formation with the small Maf protein family (Itoh, Wakabayashi et al. 1999). Neh3, the C-terminal domain, is essential for transcriptional activity of Nrf2 and it seems to have the property of recruiting putative coactivator proteins due to its protein-to-protein interaction properties (Nioi, Nguyen et al. 2005).

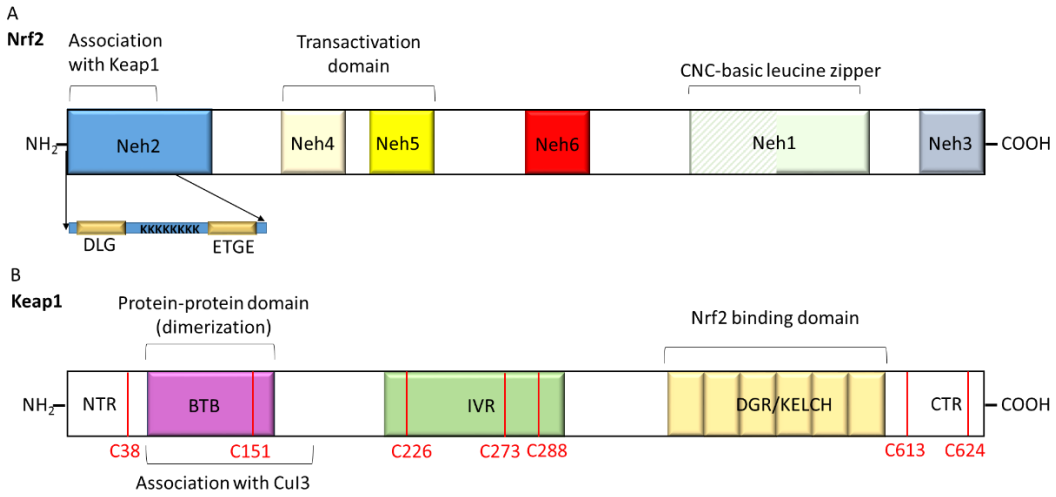

Figure 5: Nrf2 and Keap1 structure. A) Nrf2 domains. The N-terminal domain is Neh2. The Kelch domain of Keap1 binds to two binding sites located in Neh2: ETGE and DLG. The Neh4 and Neh5 are transactivation domains. Regarding Neh6, it possesses a nuclear localization signal (NLS) which is required for Nrf2 nuclear

import. The Neh1 domain contains a CNC homology region and basic-leucine zipper domain, necessary for DNA binding and heterodimer formation with the small Maf protein family. B) Domain structure of human KEAP1. NTR: N-terminal region; KEAP1 forms a homodimer through the BTB binding domain; IVR: intervening region; The Kelch domain is the binding site with Nrf2; CTR: C-terminal region. The most commonly modified cysteine residues by sulfhydryl-reactive small molecules are shown in red.

4.3. Keap1: The master regulator of Nrf2 activity

Murine Keap1 is a 624-amino-acid polypeptide that comprises five domains: the N-terminal region (NTR), the BTB (broad complex, tramtrack, and bric-a-brac) domain, the intervening region (IVR), the double glycine repeat (DGR) or Kelch domain and the C-terminal region (CTR) (reviewed by (Itoh, Tong et al. 2004, Zhang 2006)). The BTB domain is an evolutionarily conserved protein-to-protein interaction domain found in actin-binding proteins and zinc finger transcription factors. This region is involved in the homodimerization of the Keap1 protein (Zipper and Mulcahy 2002). The IVR is a cysteine-rich domain that was proven to be indispensable for Keap1 to mediate ubiquitination of Nrf2 (Zhang and Hannink 2003). The DGR or Kelch domain possesses six double glycine repeats. As mentioned above, Keap1 binds Neh2 in Nrf2 and actin through this domain (Itoh, Wakabayashi et al. 1999, Kang, Kobayashi et al. 2004). The subcellular localization of endogenous Keap1 is not well understood, but it appears to localize at the cytoskeleton in the nuclear periphery, co-localizing with F-actin (Kang, Kobayashi et al. 2004).

4.4. Nrf2 regulation

Nrf2 is not free and active all the time. Under normal conditions, Nrf2 is constantly degraded with a half-life of less than 20 minutes (Katoh, Iida et al. 2005) and thus, degradation of Nrf2 serves as a regulatory control. Nrf2 is bound to a Keap1 dimer in the cytoplasm that suppresses Nrf2-dependent transcriptional activity (Itoh, Wakabayashi et al. 1999).

The presence of the Neh2 motif is required for the repression of Nrf2 by Keap1 (Itoh, Wakabayashi et al. 1999). A two-site substrate recognition hinge-and-latch model was proposed to be the key regulatory mechanism. In this model, a Keap1 homodimer recruits Nrf2 *via* the evolutionarily conserved low-affinity DLG and high-affinity ETGE motifs within the regulatory Neh2 domain of Nrf2 (Tong, Katoh et al. 2006, Tong, Kobayashi et al. 2006, Tong, Padmanabhan et al. 2007). Keap1 binds to the actin cytoskeleton and traps Nrf2 in the cytoplasm preventing the Nrf2 nuclear translocation and its subsequent activity. It has been demonstrated that the only presence and interaction of Nrf2 and Keap1 is not sufficient to maintain Nrf2 in the cytosol but the interaction with the actin cytoskeleton is needed (Kang, Kobayashi et al. 2004). Besides its role in sequestering Nrf2 at the cytoplasm, Keap1 inhibits Nrf2 function through one additional mechanism. Keap1 acts as an adaptor

to allow the interaction between Nrf2 and the cullin3-based E3 ubiquitin ligase complex 2. The formation of this complex triggers the ubiquitylation and the subsequent turnover of Nrf2 by the proteasome (Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004).

Under stress conditions Nrf2 is released from Keap1. This dissociation occurs by two different mechanisms: direct attack by electrophiles or ROS or indirect actions such as phosphorylation (Figure 6).

4.4.1. Cysteine residues modification

Cysteine residues modification implies that Keap1 acts as a primary sensor of oxidative stress because of the presence of reactive cysteine residues. Keap1 is a relatively cysteine-rich protein since this aminoacid comprises 4.3% of all aminoacidic residues, which doubles the average number in proteins (Bryan, Olayanju et al. 2013). Cysteine residues are often observed within the functional domains of proteins and are redox-active and responsive to the local environment (Marino and Gladyshev 2012). The hypothesis that the modification of cysteines in Keap1 may be essential to the regulation of the Nrf2 pathway was first put forward when it was determined that a number of Nrf2 inducers, despite of the structural differences, were all reactive with the presence of thiol groups. In a murine model, it was shown that electrophiles were capable of dissociating Keap1 from the Neh2 region of Nrf2. Keap1 was considered as the sensor for these , as the Neh2 region contains no cysteine residues. Using mass spectrometry analysis, a number of cysteine residues were found in the IVR that were remarkably reactive (Dinkova-Kostova, Holtzclaw et al. 2002). Modifications on these reactive cysteines might induce conformational changes that trigger the dissociation of the Nrf2-Keap1 complex and allow the translocation of Nrf2 to the nucleus (Dinkova-Kostova, Holtzclaw et al. 2002, Kang, Kobayashi et al. 2004, Wakabayashi, Dinkova-Kostova et al. 2004). However, a controversy also exist about whether or not electrophiles are enough by themselves to dissociate the Nrf2/Keap1 complex. A study carried out in human cells led to the conclusion that modifications on cysteine residues are not enough for Nrf2-Keap1 complex dissociation but another mechanism such as the phosphorylation of Nrf2 is necessary (Eggler, Liu et al. 2005) (see below). Mass spectroscopy and mutagenesis analyses have established that C151 in the BTB domain, and C273 and C288 in the IVR domain are critical for the repressor function of Keap1. However, depending on the reaction conditions and the experimental system used, other cysteine residues have been also reported to be modified by sulforaphane, such as C38 in the N-terminal domain, C368, C489, and C583 in the Kelch domain, and C624 in the CTR (reviewed by (Dinkova-Kostova 2012)).

Nitric oxide (NO), a signaling molecule involved in a number of physiological processes has also been demonstrated to induce heme oxigenase-1 (HO-1) gene transcription through the activation of the Nrf2/ARE complex in smooth muscle cells, inducing the release of Nrf2 from Keap1 (Liu, Peyton et al. 2007). Experiments showing that macrophages from Nrf2 knockout mice exposed to exogenous or endogenous NO are unable to up-regulate sulfiredoxine support the idea of a NO-dependent Nrf2 regulation (Abbas, Breton et al. 2011).

4.4.2. Nrf2 phosphorylation

Several protein kinase pathways have been implicated in transducing oxidative stress signals to gene expression mediated through the ARE. A critical step in the signaling cascade that leads to ARE activation might be the phosphorylation of Nrf2 by protein kinase C (PKC) (Huang, Nguyen et al. 2000). The phosphorylation site for PKC is Ser-40 (Huang, Nguyen et al. 2002). *In vitro* studies have shown that phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1 (Huang, Nguyen et al. 2002, Bloom and Jaiswal 2003, Numazawa, Ishikawa et al. 2003). In addition, it has been shown that the accumulation of unfolded proteins in endoplasmic reticulum (ER) activates Nrf2 *via* the direct phosphorylation of Nrf2 by ER-localized pancreatic endoplasmic reticulum kinase (PERK) (Cullinan, Zhang et al. 2003). It is suggested that the disruption or rearrangement of actin cytoskeleton by the Phosphatidylinositol 3-kinase (PI3K) triggers Nrf2 activation and translocation to the nucleus (Kang, Lee et al. 2002).

More recently, PI3K and its downstream target Akt have been also linked to activation of ARE-mediated gene expression in hepatoma and neuroblastoma. In addition, a number of reports have addressed a possible role for extracellular signal-regulated kinase (ERK1/2) in ARE activation. However, these findings have remained controversial: ERK1/2 has been found to positively regulate the ARE in certain hepatoma cells but negatively in other cell types. Similarly, p38 MAPK (mitogen-activated protein kinase) has also been shown to affect ARE activity either positively or negatively (reviewed by (Huang, Nguyen et al. 2000)). Phosphorylation of Nrf2 by p38 produces different outcomes depending on the model of study. In some cases, p38 has been reported to promote the dissociation of Nrf2 from the Nrf2–Keap1 complex, either directly by phosphorylating Nrf2 or indirectly through intermediary kinases, resulting in greater Nrf2 accumulation in the nucleus and the activation of some antioxidant target genes (Martin, Rojo et al. 2004). However, other studies have described a different response with no role for p38 on Nrf2 activation (Li, Wu

et al. 2011) or even the inhibition of Nrf2 transcriptional activity by p38 (Shen, Hebbar et al. 2004, Song, Li et al. 2014).

These data support the interesting possibility that cell signaling other than redox regulation can also regulate the Nrf2 pathway induction. Thus, more complex mechanisms might lead to the Nrf2-mediated cell survival response.

Once Nrf2 is released from Keap1 by any of these mechanisms, Nrf2 can be imported into the nucleus (Dinkova-Kostova, Holtzclaw et al. 2002, Wakabayashi, Dinkova-Kostova et al. 2004). Nrf2 in the nucleus heterodimerizes with a group of bZip proteins termed small musculoaponeurotic fibrosarcoma proteins (small Maf) (Itoh, Igarashi et al. 1995). Nrf2 requires a member of the small Maf proteins as an obligatory partner molecule for binding to the DNA sequence. The dimerization of Nrf2 with small Maf stimulates the transcription of the downstream targets by improving the specificity to bind to a *cis*-acting enhancer of the ARE contained in the promoters of these genes (Friling, Bensimon et al. 1990, Itoh, Chiba et al. 1997, Yamamoto, Kyo et al. 2006).

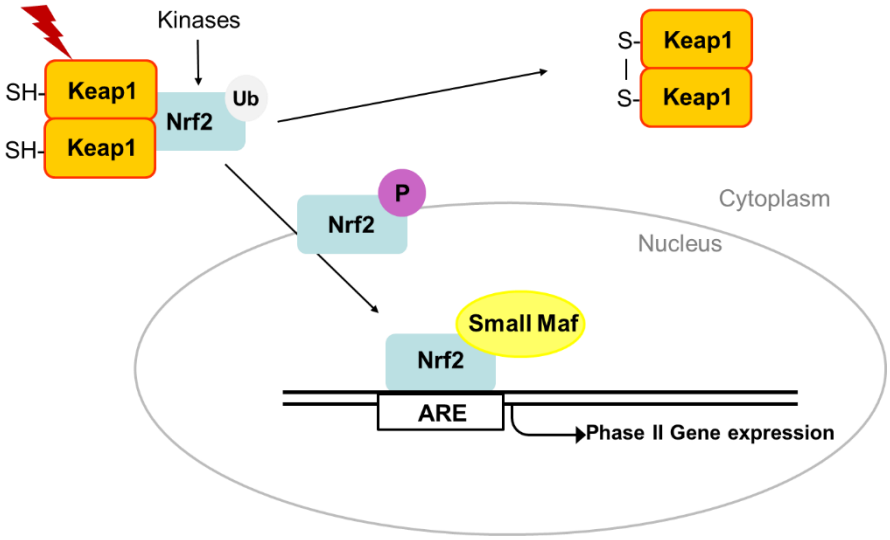

Figure 6: Mechanism of Nrf2/Keap1 pathway induction. Nrf2 is anchored in the cytoplasm by binding to Keap1, which is attached to the actin cytoskeleton. Inducers disrupt the Keap1–Nrf2 complex, and then Nrf2 migrates to the nucleus where it forms heterodimers with co-activator proteins such as small Maf that bind to the ARE regions of phase 2 genes and accelerate their transcription.

4.5. Functions of Nrf2

4.5.1. Nrf2 and oxidative stress response

Under physiological conditions Nrf2-dependent signaling is an oxidative and electrophilic stress sensor acting as a switch that is turned on by the presence of stressors in the cellular environment. A set of genes encoding detoxifying and antioxidative stress proteins are induced in a coordinated way in response to electrophiles and ROS by Nrf2 through ARE (Friling, Bensimon et al. 1990, Rushmore, Morton et al. 1991). Genes encoding a subset of drug metabolizing enzymes, such as glutathione S-transferases (GSTs) (Friling, Bensimon et al. 1990) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (Rushmore, Morton et al. 1991), have been shown to be under ARE/EpRE regulation, along with a subset of antioxidant genes, such as HO-1 (Alam, Camhi et al. 1995), the subunits of g-glutamylcysteine synthetase (g-GCS) (Mulcahy, Wartman et al. 1997), and Trx (Kim, Masutani et al. 2001).

Homozygous Nrf2 knockout (KO) mice are normal in appearance and showed no difference in their development compared with their wild-type littermates (Chan, Lu et al. 1996). However, these mice show lower levels and no induction of phase II detoxifying enzymes and the wound repair process was also reduced (Braun, Hanselmann et al. 2002). These mice are also extremely susceptible to the administration of the antioxidant butylated hydroxytoluene, causing death in these animals from acute respiratory distress syndrome. Nrf2 knockout mice are also highly sensitive to acetaminophen-induced hepatotoxicity, mostly due to the depletion of GSH and the impairment of its *de novo* synthesis (reviewed by (Braun, Hanselmann et al. 2002). Nrf2 disruption enhances susceptibility to oxidative stress and alters cell cycle progression, which has been explained on the basis of GSH (Reddy, Kleeberger et al. 2008). In addition, older mice develop a severe autoimmune disease resembling systemic lupus erythematosus (Li, Stein et al. 2004). Loss of Nrf1 and Nrf2 leads to marked oxidative stress in cells that is indicated by elevated intracellular reactive oxygen species levels and cell death that is reversed by culturing under reduced oxygen tension or by the addition of antioxidants. These double KO mice show embryonic lethality as well (Leung, Kwong et al. 2003).

4.5.2. Nrf2 and apoptosis

A crosslink between Nrf2 and several effectors of apoptosis has been proposed. Tumor suppressor p53, which induces apoptosis upon DNA damage and is partially regulated by ROS, inhibits transcriptional activation of Nrf2. Gañán-Gómez and colleagues have reviewed several studies regarding the link between Nrf2 and p53. Taken together, these reports

indicate that p53-dependent apoptosis induced by several stressors (genotoxic and oxidizing agents) requires the inhibition or silencing of Nrf2 and subsequently, the Nrf2-regulated antioxidant genes. Therefore, an increase in the expression of the antioxidant gene battery could interfere with p53-dependent apoptosis induced after DNA damage and thus favor the development of cancer cells (reviewed by (Ganan-Gomez, Wei et al. 2013)).

Another important connection between Nrf2 activity and apoptotic pathways is the Nrf2-mediated regulation of proteins from the Bcl-2 family, like Bcl-2 and Bcl-xL (Chen, Li et al. 2012, Niture and Jaiswal 2012, Niture and Jaiswal 2013). Previous investigations have demonstrated that the expression of antiapoptotic Bcl-2 and Bcl-xL proteins is under direct transcriptional control of Nrf2 because of the presence of an ARE (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Also, activation of Nrf2-dependent transcription results in drug resistance and cell survival while its inhibition is linked to the potentiation of etoposide and radiation-induced apoptosis in mouse and human hepatic tumor cells (Niture and Jaiswal 2012). Stabilization of Nrf2 in the nucleus also increased the levels of antiapoptotic proteins Bcl-2 and Bcl-xL and resulted in cardioprotective effects in mice subjected to myocardial ischemia/reperfusion (Calvert, Jha et al. 2009). In the context of aging, it has been shown that the impact of Nrf2 deletion on skeletal muscle is increased in aged animals, resulting in higher expression of different apoptotic markers such as caspases-3 and -9, Bax, Bad, and AIF (Miller, Gounder et al. 2012).

4.5.3. Nrf2 and tumorigenesis

Increased oxidative stress and DNA damage situations are produced when Nrf2 is disrupted. Nrf2 loss has been related with the initiation of cellular transformation in prostate cancer (Frohlich, McCabe et al. 2008). On the same way, low levels of Nrf2 activity predispose cells to chemical carcinogenesis, most likely because of a poor detoxification. Of note, Nrf2KO mice are not protected against carcinogenesis by calorie restriction (Pearson, Lewis et al. 2008). Moreover, a regulatory single nucleotide polymorphism in the NRF2 gene which results in decreased gene expression is linked to human lung cancer susceptibility (Suzuki, Shibata et al. 2013). Conversely, upregulation of Nrf2-mediated signaling has been shown to have protective effects against different types of cancer (Giudice and Montella 2006, Jeong, Jun et al. 2006). In a 4-nitroquinoline-1-oxide (4NQO)-induced carcinogenesis of the upper aerodigestive tract model, Nrf2KO mice showed more susceptibility to carcinogenesis. Conversely, Nrf2-overexpressing mice were more resistant to develop carcinogenesis (Ohkoshi, Suzuki et al. 2013). Thus, Nrf2 has been recognized as a tumor suppressor gene (Martin-Montalvo, Villalba et al. 2011) and activation of Nrf2 and its

downstream targets may constitute a potentially valuable therapeutic strategy to treat oxidative stress-induced diseases, including cancer (Suzuki, Motohashi et al. 2013)

However, there is a dark side of Nrf2 function; and Nrf2 has been also considered as a proto-oncogene (Wang, Sun et al. 2008, Suzuki, Motohashi et al. 2013) because uncontrolled activation of Nrf2 may allow the survival of potentially dangerous damaged cells leading to oncogenesis and chemoresistance. This specially applies to tumor cells with loss-of-function mutations in the *Keap1* gene, as found in several lung cancer cells, that abolish proper interaction of the Keap1 protein with Nrf2 leading to persistent nuclear accumulation of Nrf2 and activation of antioxidant and anti-apoptotic gene expression thus favoring drug resistance (Ganan-Gomez, Wei et al. 2013). Therefore, Nrf2 can be a good target for pharmacological inhibition in cancer. The use of inhibitors such as retinoic acid receptor α agonists or brusatol could be a therapeutic tool, even though many more studies need to be carried out. Our research supports that the Nrf2 inhibition would favor cell growth arrest and sensitize cancer cells to apoptosis (Wang, Sun et al. 2008, Suzuki, Motohashi et al. 2013) and also sheds some light on the putative underlying mechanisms.

5. The two-electron quinone reductase NQO1

Quinones are a group of natural compounds widely distributed in bacteria, fungi, plants and animals. They appear as polycyclic aromatic compounds formed by various combustion processes, e.g. cigarette smoke and diesel and automobile exhausts (reviewed by (O'Brien 1991)). Quinone reductases can be classified into two categories according to their mechanism of quinone reduction (reviewed by (Deller, Macheroux et al. 2008)).

a) One-electron quinone reductases, which reduce quinones by two sequential steps of one electron donation, leading to the generation of potentially harmful semiquinone species. Besides the mitochondrial quinone reductases, two integral membrane protein use this mechanism: cytb5R and NADPH-cytochrome P450 reductase. The instability of semiquinone radicals leads to their reaction with molecular oxygen. As a result of this reaction the oxidized quinone is regenerated by redox cycling but there is also a production of superoxide. Oxidized quinones can be subsequently reduced again to semiquinones, and the spurious cycle repeated.
b) Two-electron quinone reductases, which catalyze the simultaneous transfer of two electrons and two protons to the quinone substrates, yielding hydroquinones and avoiding the generation of unstable semiquinones. The two-electron reduction of quinones and their derivatives prevent the deleterious effects of redox cycling.

Instead, the hydroquinones produced by two-electron reduction are more stable chemicals which can then be conjugated with glutathione or glucuronic acid for their rapid excretion.

The two electron reductases are flavoproteins that can be found in both prokaryotic and eukaryotic organisms, conveying the importance of this system. They were initially named as DT-diaphorases because they can use both di- or tri-phosphoryridin nucleotides, DPNH (NADH) and TPNH (NADPH) respectively, as the electron donors with almost equal affinity. Eventually, the term diaphorase started being used to describe any dehydrogenase enzyme, mainly a flavoprotein. In order to avoid any confusion, the first DT-diaphorase described by Ernster and Navazio in 1958 (Ernster and Navazio 1958) was referred to as NAD(P)H:quinone oxidorreductase 1 (NQO type 1, NQO1, EC 1.6.99.2) (Figure 7).

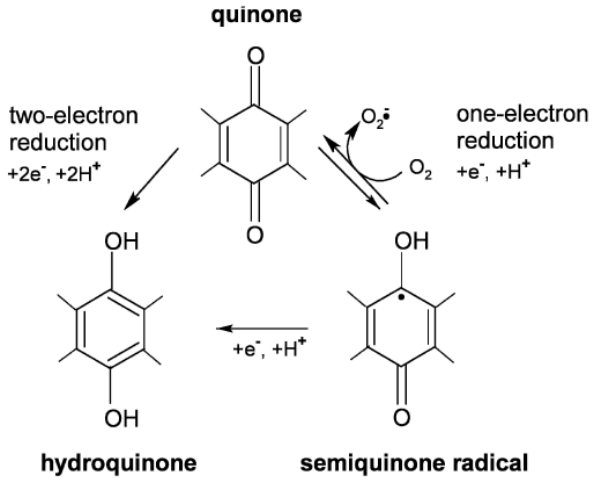

Figure 7: Reduction mechanism of quinones. Formation of a hydroquinone is achieved by the two-electron reduction process, whereas the one-electron reduction of quinones supports the formation of a semiquinone radical intermediate. Figure taken from (Deller, Macheroux et al. 2008)

NQO1 is a flavoprotein that catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones, using either NADH or NADPH as the electron donor. NQO1 carries out its catalytic function by a ping-pong mechanism. In a first step, NAD(P)H binds to NQO1 to create a NQO1-NAD(P)H complex. Subsequently a FAD molecule is reduced by NAD(P)H to generate $FADH_2$. The exit of $NAD(P)^+$ from the catalytic site allows the substrate to bind, the electron transfer from FADH to the substrate and the release of the reduced product (Li, Bianchet et al. 1995).

5.1. NQO1 gene: characteristics and expression

The *NQO1* gene is located in the chromosome 16q22.1. This gene has a length of 20kb and consists of six exons and five introns. Several directed mutagenesis experiments have shown three *cis*-elements in the promoter region which are responsible for its basal expression and induction: the ARE, the xenobiotic responsive element (XRE) and an AP-2 element that is required for the NQO1 induction by cyclic adenosine monophosphate (cAMP). ARE is needed for its basal expression and induction by several types of compounds. The induction of NQO1 by ARE has been observed under oxidative stress situations and upon exposure of cells to xenobiotics, ultraviolet radiation and hypoxia.

There are several transcription factors that are able to bind to the ARE element to regulate the expression of NQO1 such as c-Jun, Jun-B, Jun-D, c-Fos, Fra1, Nrf1 y Nrf2. The main factor is Nrf2, whose absence in Nrf2 knockout mice decreases significantly the expression and induction of NQO1 (reviewed by (Jaiswal 2000)).

There are three different transcripts of NQO1 with 2.7, 1.7 and 1.2 Kb because of the existence of different polyadenylation sites ((Jaiswal, McBride et al. 1988)). A shorter fourth transcript (1.1 kb) generated by alternative splicing has been identified in human cancer cell lines. This truncated transcript codifies for a protein lacking exon 4, where the putative quinone binding site is located. Even though the truncated protein has been expressed in *E. coli*, it has not been found in human cells. This suggests that the messenger RNA that undergoes alternative splicing does not undergo translation (Gasdaska, Fisher et al. 1995). Alternative splicing can be a regulatory mechanism of NQO1 in humans where the constitutive proportion between full length transcripts and short transcripts is approximately 90:10 (Pan, Han et al. 2002).

5.2. NQO1 protein

The product of the NQO1 gene is an enzyme of 274 residues. The crystal structure of protein NQO1 has been determined in three different mammals: rat (PDB D4A), human (PDB 1D4A) and mouse (PDB 1DXQ), as well as NQO1-substrate complexes with different chemotherapeutic agents such as duroquinone (PDB 1DXO, (Faig, Bianchet et al. 2000)), (PDB 1H66), EO9 (1GG5) and ARH019 (1H69) (Faig, Bianchet et al. 2001) and inhibitors with different grades of specificity towards NQO1 such as dicoumarol (PDB 2F1O, (Asher, Dym et al. 2006)), ES936 (PDB 1KBQ, (Winski, Faig et al. 2001)) or Cibacron Blue (PDB 1QRD, (Li, Bianchet et al. 1995)).

The functional NQO1 unit is a homodimer. Extensive contact between monomers confers high stability to these dimers, having 265 monomer-monomer contacts shorter than 4 Å. Each monomer possesses two domains: a flavodoxine-like catalytic domain of 220 residues and a C-terminal domain of 54 residues. FAD molecules are non-covalently bound to each monomer. Their isoalloxazine rings lay at one side of two equivalent crevices present at opposite ends of the monomer-monomer interface. These crevices form two identical catalytic sites that work independently. One side of the isoalloxazine of the FAD molecule of one of the monomer make one side of the catalytic domain. The other side is formed by residues of the catalytic domain of the other monomer. The size and shape of the catalytic site allows the docking of a variety of cyclic compounds. The catalytic site contains a pocket of 360 Å containing a residue of tryptophan and phenylalanine (Trp105 and Phe106) at the bottom. Two Tyr residues from the other catalytic domain (Tyr 126' and Tyr 128') are located above the FAD molecule to make the roof of the pocket and establish polar interaction with the substrates. The access to the catalytic site is limited by four residues (Gly 149, Gly150, His 194 and Pro 68). One residue, His 161, performs the main role in the catalytic mechanism of the enzyme. Finally the Tyr 128 residue gates the pocket and protects the isoalloxazine of the FAD from oxygen attack (reviewed by (Bianchet, Faig et al. 2004)).

The ping-pong mechanism for the obligatory two-electron reaction consists of two direct hydride transfer: one from the NAD(P)H to FAD and the second one from the reduced FAD ($FADH_2$) to the substrate electron receptor (Figure 8). The electron donor and acceptor share the same site in the enzyme so the binding of the acceptor substrate needs the release of the $NAD(P)^+$. Several studies of site-directed mutagenesis have shown that none of the single mutations that have been studied affect to a great extent on the NQO1 activity. This can be explained by the direct transfer of hydride groups between the flavin, NAD(P)H and the quinone. The protein might be the mediator to place them on the right site and to minimize the negative effects of charge separation. There is a number of residues that cooperate in these two functions, in such a way that the enzyme shows a great redundancy and single mutations (except for His 161) do not influence its catalytic activity (reviewed by (Bianchet, Faig et al. 2004))

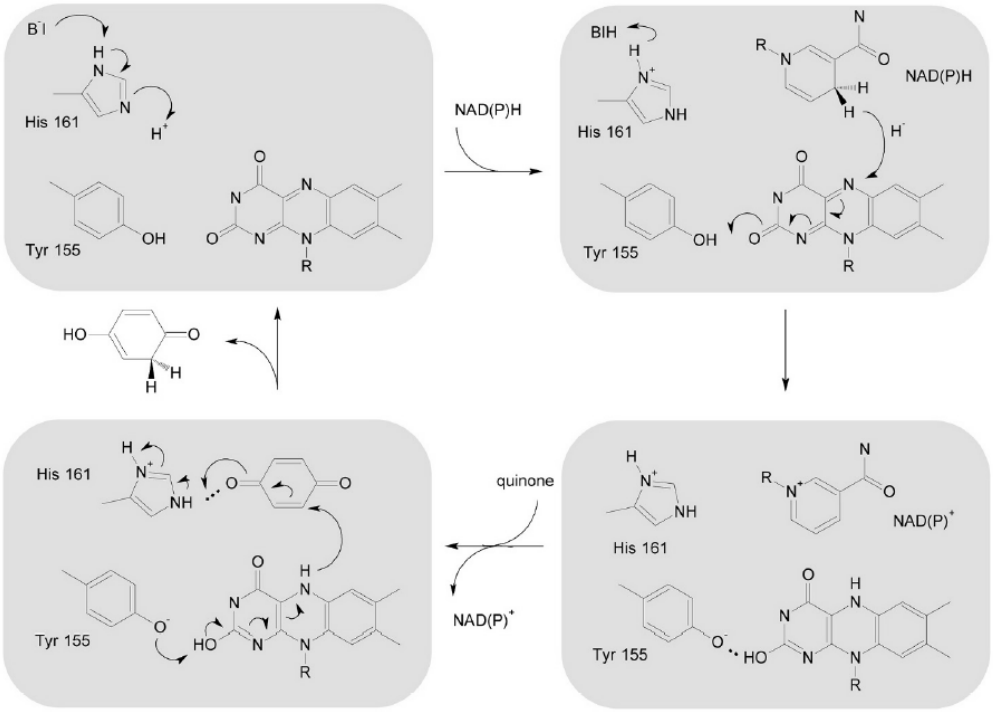

Figure 8: Reaction mechanism of human NQO1. The substrate reduction process has been proposed to occur *via* two direct hydride transfer steps: the first one from the electron donor (NAD(P)H) to the flavin cofactor (FAD or FMN) and the second one from the reduced flavin to the quinone acceptor. Figure taken from (Deller, Macheroux et al. 2008).

The tridimensional structure study of NQO1 shows a great plasticity in the binding site of the enzyme. This allows for the interaction of a variety of substrates such as antitumor quinones (Faig, Bianchet et al. 2001) or inhibitors like dicoumarol and ES936 (Winski, Faig et al. 2001, Asher, Dym et al. 2006). The number of quinonic substrates includes molecules of different sizes such as benzoquinone, menadione or mitomycin C. This is important for the design of chemotherapeutic compounds that could be bioactivated by NQO1 (Faig, Bianchet et al. 2001).

5.3. Intracellular distribution of NQO1

NQO1 is mainly a cytosolic protein, but it has also been found in the nuclei of human tumor cells HT29 and H661 (colon and lung, respectively) using confocal and immunoelectron microscopy (Winski, Koutalos et al. 2002), and by western-blot in MMRU melanoma human cells transfected with a plasmid containing NQO1 (Garate, Wong et al. 2008). This location has implications for the bioactivation of antitumor quinones by NQO1 to induce DNA damage. For instance, the toxicity of mitomycin C increases upon overexpression of NQO1 in the nuclei of CHO cells (Seow, Penketh et al. 2004).

For efficient passive diffusion through the nuclear pore, a protein should be less than 40-60 kDa in size and, more importantly, <10 nm in diameter. NQO1 is not able to go through the nucleus pore complex by diffusion because of its size (60-70 nm diameter) and molecular weight of 61 kDa (two monomers of 30.7 kDa each). Furthermore, NQO1 does not possess any nuclear localization sequence. However, NQO1 has two heat shock protein (Hsp) 70 binding sites that could explain its nuclear location because Hsp70 is known to chaperone proteins into cellular compartments, including the nucleus (reviewed by (Winski, Koutalos et al. 2002)).

NQO1 has also been detected in the mitochondria and initially, a putative function for NQO1 in mitochondrial respiration was proposed (Conover and Ernster 1962). However, others authors were not able to confirm this observation (Winski, Koutalos et al. 2002) and techniques that preserve the integrity of cellular components such as confocal and immunoelectronic microscopy did not corroborate the presence of NQO1 in the mitochondria. .

5.4. NQO1 polymorphisms

The study of NQO1 deficiency in different human tumors led the investigators to find a mutation in the exon 6 of the gene *NQO1*. The substitution of the cytosine in position 609 by a thymine (C609T) leads to the change of the 187 proline residue by a serine (Traver, Horikoshi et al. 1992). This mutation has been denoted as the genetic polymorphism *NQO1*2*. The cell lines and tissues of organisms that have been genotyped as homozygous for *NQO1*2* do not present NQO1 activity. Some of the cell lines used in research which are homozygous for the *NQO1*2* polymorphism are BE and Caco-2 of colon carcinoma, MDA-MB-486 and MDA-MB-231 of breast cancer and NCI-H1570 and NCI-H596 of lung cancer (reviewed by (Ross and Siegel 2004)). This mutation does not affect transcription and translation of NQO1*2 and the lack of activity is explained at a post-translational level. In fact, it has been clearly established that the NQO1*2 polypeptide undergoes ubiquitylation and is rapidly degraded by the proteasome. The mean life of this polypeptide is around 1.2 hours. The substitution of the proline by serine at position 187 leads to conformational changes that could explain the instability of the polypeptide (Traver, Horikoshi et al. 1992). Also, it has been shown that the chaperone Hsp70 interact with the non-mature forms of NQO1 but not with the NQO1*2 polypeptide. This lack of interaction could lead to an incorrect folding and the subsequent ubiquitylation and proteasome-mediated degradation (Anwar, Siegel et al. 2002).

The allelic frequency of the *NQO1*2* polymorphism is 0.49 within Chinese population, 0.46 for the Inuits, 0.4 in the Native Americans and 0.16 within Caucasians. The homozygous genotype *NQO1*2/*2* which completely lacks NQO1 activity is found in the 22% of the population in Asia. These individuals are more sensitive to the benzene hemotoxicity and show a higher risk of undergo cancer, specifically leukemia (Rothman, Smith et al. 1997, Ross and Siegel 2004).

Finally, the polymorphism *NQO1*3* has been also characterized. In this polymorphism the cytosine 465 is substituted by a thymine (C465T) in a codon encoding for a tryptophan residue instead of an arginine. The mutated protein, as opposed to NQO1*2, is as stable as the non-mutated version, but its enzymatic activity varies depending on the substrate. NQO1*3 reduced menadione at the same ratio but its ability to metabolize mytomicin C is reduced by 60% (Pan, Forrest et al. 1995, Pan, Han et al. 2002). The allelic frequency of this polymorphism is very low: 0.05 in Caucasians, 0.04 in Chinese population and 0.01 in Native Americans (reviewed by (Ross and Siegel 2004))

5.5. NQO1 functions

NQO1 inhibitors have been used to elucidate the role of NQO1 in physiology. The pharmacological inhibition was based on the specificity of the inhibitor, sometimes with no final evidences. A number of substances have been described as NQO1 inhibitors, mainly those compounds competing with NAD(P)H for the active site of the enzyme. Among all of them, the most potent is dicoumarol [3,3'-methylenbis(4-hidroxicumarine)], used from the fifties as an oral anticoagulant, whose Ki is 1-10nM in *in vitro* assays. Another coumarins such as warfarin, used as an oral anticoagulant as well, or esculetin (Garten and Wosilait 1971), flavons like phenidone, chrysin or 7,8-dihidroxiflavone (Chen, Wu et al. 1999), the colorant Cibacron Blue (Prochaska 1988) and the curcumine (Tsvetkov, Asher et al. 2005), a natural phenolic compound used as a food colorant in curry have been described as competitive inhibitors of NQO1, but less potent that dicoumarol.

The indolquinone ES936 (5-metoxi-1,2-dimethyl-3-[(4-nitrofenoxi)methyl]indol-4,7-dione) has been also described as an irreversible inhibitor of NQO1 (Winski, Faig et al. 2001). The inhibition of NQO1 by ES936 is NAD(P)H dependent and a concentration of 100 nM is enough to inhibit more than the 95% of the NQO1 activity of several cell lines in 30 minutes. It has been noted that other cellular reductases were not inhibited and the levels of thiols were not altered even though the dose was high enough to induce DNA strand breaks (Dehn, Siegel et al. 2003). However, more recent studies have shown that ES936 inhibits the TrxR activity, being its cytotoxic action on pancreatic tumor cells not only

accounted by the inhibition of NQO1 (Yan, Shieh et al. 2009). New ES936-derived compounds have been developed with a more specific effect on NQO1 and a lower cytotoxic action (Colucci, Reigan et al. 2007).

The study of the inhibition of NQO1 has been extended to the development of cell lines and organisms that genetically lack *NQO1*. From the discovery of NQO1, this enzyme has shown its functional versatility, being an excellent example of economy in the cell physiology (Dinkova-Kostova and Talalay 2010). The functions of NQO1 that have been described so far are:

5.5.1. Quinone reductase

This is the function classically conferred to NQO1. There is a reduction of quinones to hydroquinones by transferring two electrons and using both NADH and NADPH as cofactor substrates. This two-electron reaction prevents the generation of reactive semiquinones, and at the same time regulates the NAD(P)+/ NAD(P)H intracellular ratio. The presence of NQO1 protects from the cytotoxic, carcinogenic and mutagenic effects of a number of quinone compounds.

NQO1KO mice have higher levels of lipid peroxidation and DNA damage than wild-type mice when exposed to menadione (an analog of vitamin K), to BPQ ((benzo[*a*]pyrene-3,6-quinone, a metabolite of benzo[*a*]pyrene) or to benzoquinone (a metabolic product of benzene) (Joseph, Long et al. 2000). These mice are also prone to develop skin tumors induced by DMBA (7,12-dimethylbenzantracene) a mutagenic compound that is generated by the incomplete combustion of fossil fuel and is used in research as an initiator for the carcinogenesis process (Long, Waikel et al. 2001). The tissue distribution of NQO1 is coherent with its detoxifying function. In mice, rats and humans NQO1 is mainly found in epithelia and endothelia, facilitating that compounds entering the organism can interact with that enzyme (Siegel and Ross 2000).

5.5.2. Bioactivator enzyme

The reduction of compounds carried out by NQO1 does not always implies the elimination of the damaging nature of a substrate. On the contrary, in some circumstances the completely reduced form of the quinone (hydroquinone) can be more deleterious that the oxidized form. The detoxification is efficient if facilitates the excretion of the hydroquinone, either by increasing its solubility in water or by sulfonation or glucuronidation. However, contrary to the established dogma that the two electron-reaction mechanism yield redox-

stable hydroquinones, some hydroquinones can autooxidize and generate ROS or rearrangement of hydroquinones to alkylating agents (Cadenas 1995).

Since it has been shown that several human solid tumors express high levels of NQO1, the bioactivator capacity of this enzyme has been used to test different compounds as anti-tumor agents. This pro-drugs compounds are transformed into cytotoxic agents after being reduced. The overexpression of NQO1 in tumor cells allows that the toxic action of the antitumor agent is concentrated in the tumor cells and not in healthy cells with lower levels of NQO1.

In this way, different quinone compounds have been tested such as mytomicin C, RH1 (a water-soluble form homolog of dimethylaziridinylbenzoquinone), EO9 and ARH109 (both aziridinylindoquinones) (reviewed by (Alcain and Villalba 2007)). The presence of NQO1 in the nuclei of cancer cells makes the bioactivator action of this enzyme more interesting in order to trigger damage in the DNA (Winski, Koutalos et al. 2002).

5.5.3. Antioxidant enzyme

In addition to the activation and detoxification of exogenous compounds, it is important to consider the involvement of NQO1 in the antioxidant metabolism of molecules such as ubiquinone (coenzyme Q) and vitamin E. Both molecules have a long hydrophobic tail that localize them to the hydrophobic core of biomembranes and lipoproteins, resulting in a protection of these structures against lipid peroxidation. The reduction of the ubiquinone by NQO1 generates ubiquinol, a highly antioxidant compound. The interaction of the tocopheroxyl radical of the vitamin E with free radicals causes its oxidation. The antioxidant form (α-tocopherol) can be regenerated by NQO1 in an indirect way in a ubiquinone dependent process (Bello, Kagan et al. 2003). On the other hand it has been demonstrated that the α-tocopherylquinone, a derivative of the oxidation of α-tocopherol, can be reduced to α-tocopherylhydroquinone in a reaction catalyzed by NQO1 (Siegel, Bolton et al. 1997).

5.5.4. $O_2^{\cdot-}$ scavenger

During the course of one study focused on the auto-oxidation of NQO1 it was shown that the stoichiometric relation between NADH oxidation and O_2 consumption is approximately of 1:1. This auto-oxidation can be accelerated in the presence of $O_2^{\cdot-}$ and be inhibited by the addition of SOD. It seems to follow the pattern of auto-oxidation of flavins (Siegel, Gustafson et al. 2004). FAD cofactor is not covalently bound to NQO1, but it is maintained by hydrogen bonds and van der Waals forces to the active site (Faig, Bianchet et al. 2000). In the *in vitro* assays, superoxide scavenger activity of NOQ1 ceases when ES936 is added. This

demonstrates that NQO1 scavenger activity depends on its catalytic activity (Siegel, Gustafson et al. 2004). The *in vivo* relevance of this activity remains to be elucidated. In any case, the reaction kinetics suggests that superoxide elimination by NQO1 is less than one order of magnitude higher than the rate of chemical dismutation of O_{2^-} and around four orders of magnitude lower than the rate of enzymatic dismutation (Siegel, Gustafson et al. 2004). Therefore, the scavenger activity of NQO1 might be only significant in the assumption of a very high intracellular concentration. Since NQO1 is induced under oxidative stress situations, it is possible that in these conditions the scavenger activity is part of the overall antioxidant defense. There are not many studies about the real biological importance of this role of NQO1. There are registers of NQO1 scavenger activity in cytosolic extracts of rat aortic smooth muscle A10 and H9c2 cells and human aortic smooth muscle cells treated with D3T (3H.1,2-dithiol-3thione), a NQO1 inducer in these cells. These cell lines have lower levels of SOD, so the induction of NQO1 can be a good defense from superoxide (Zhu, Jia et al. 2007).

5.5.5. Interactions with other proteins

During the last years new implications for NQO1 apart from its quinone reductase activity have been studied. NQO1 binds the tumor suppressor protein p53 stabilizing it. This interaction is reinforced in the presence of NADH while the addition of NQO1 inhibitors induced the proteosomal degradation of p53 and inhibited p53-dependent apoptosis (Asher, Lotem et al. 2001, Tsvetkov, Reuven et al. 2010). p53 is a short-life protein and its cellular levels are controlled by its proteosomal degradation. This is achieved mainly by the Mdm-2 protein that associates with p53 and, by virtue of its ubiquitin ligase activity, targets p53 for proteosomal degradation by proteasome 26S (the 20S catalytic core and two 19S regulatory subunits). However, NQO1 regulates the degradation of p53 without the interaction of Mdm-2 in the 20S proteosome (the catalytic core of the 26S proteasome). Experiments carried out in human colon carcinoma cells overexpressing NQO1 show higher levels of p53 compared to the original cells. Genetic silencing of NQO1 by RNAi reduced levels of p53 (Asher, Lotem et al. 2002). NQO1 also participated in the p53 accumulation after γ-radiation exposition or genotoxic agents (Asher and Shaul 2005). In the same way, a role of NQO1 in p53 accumulation under oxidative stress conditions has been also described. Thus, the H_2O_2 treatment induced a higher accumulation of p53 in cells overexpressing NQO1 than the original cells (Asher, Lotem et al. 2002). The link between NQO1 and p53 suggests that redox reaction controlled by oxidoreductases like NQO1 can be a decisive factor in the intracellular levels of p53 and subsequently, tumor development.

The study of interactions between p53 and NQO1 with NQO1 inhibitors have allowed to better understand this association. Treatment with ES936, an irreversible inhibitor that blocks the catalytic function of NQO1, has no effect on its interaction with p53 and the stability of this tumor suppressor. However, dicoumarol inhibits the association of p53 and NQO1. Dicoumarol displaces NAD(P)H cofactor, inhibiting not only the catalytic activity but also the interaction with p53. Curcumine has the same effect (Tsvetkov, Asher et al. 2005). These results, together with the obtained by co-immunoprecipitation (Anwar, Dehn et al. 2003), confirm that p53 and NQO1 have a physical interaction that requires the presence of NAD(P)H but does not require NQO1 catalytic activity.

The fact that NQO1 participates in the stabilization of p53, a tumor suppressor protein, can indicate a close link between NQO1 and tumor development. In this way, there is a higher susceptibility to undergo tumorigenesis in NQO1KO mice and people with the polymorphic *NQO1*2* form (Lafuente, Casterad et al. 2000, Long, Waikel et al. 2000). This polymorphic form, in addition to the lack of catalytic activity, is unable to stabilize p53 (Asher, Lotem et al. 2002). It is not clear yet whether the predisposition to develop cancer is due to the lack of NQO1 activity – and the subsequent impairment of antioxidant response – or to the destabilization of p53. For instance, presence of *NQO1*2* polymorphism is a robust predictive factor in breast cancer (Fagerholm, Hofstetter et al. 2008). For the hepatocellular carcinoma it has been suggested as a very valuable factor for the prognosis of the evolution of this cancer (Chiu, Ko et al. 2009). The association may be of great importance if we consider that approximately 4% of Caucasians and 20% of the Asians are homozygous for *NQO1*2* (Ross and Siegel 2004).

It is known that NQO1 also regulates other members of the p53 family like p73 (Asher, Lotem et al. 2002) and p63 (Hershkovitz Rokah, Shpilberg et al. 2010). NQO1 also binds to Hsp70 and Hsp40. Hsp70 interacts with the early form of NQO1, probably intervening in the folding. It has not been registered any interaction between Hsp70 and NQO1*2, suggesting that the incorrect folding of the NQO1*2 facilitates its ubiquitylation and subsequent degradation (Anwar, Siegel et al. 2002). Also this interaction could explain the translocation of native NQO1 from cytosol to nucleus (Winski, Koutalos et al. 2002).

NQO1 also interact with the ornithine decarboxylase (ODC) and with $p33^{ING1b}$. ODC is an enzyme that catalyzes the decarboxylation of the ornithine to generate putrescine, the first compound in the biosynthetic pathway of the polyamines. Polyamines are essential for cell proliferation and are involved in several processes like differentiation or apoptosis. Intracellular levels of ODC and activity are highly controlled because, even though they are

indispensable for the cell growth, at high levels they produce cytotoxicity. In fact, transformed and tumor cells have normal levels of polyamines (Pegg 1988, Gerner and Meyskens 2004). Degradation of ODC by antizyme is 26S proteasome-mediated. However, as also reported for p53 and its family of protein, ODC can be degraded by 20S proteasome without requiring the previous binding of ubiquitin. Interaction of ODC with NQO1 stabilizes ODC and prevents its degradation in the 20S proteasome (Asher, Bercovich et al. 2005, Asher and Shaul 2005).

NQO1 also interacts with $p33^{ING1b}$. This nuclear protein is a member of the inhibitor of growth family (ING). Low levels of $p33^{ING1b}$ can be found in some carcinomas and its overexpression induced the cell cycle detention. This protein is part of the cell response to different types of stress and its expression depends on p53 (Campos, Chin et al. 2004). NQO1 inhibits degradation of $p33^{ING1b}$ by the 20S proteasome (Garate, Wong et al. 2008). Since $p33^{ING1b}$ is a nuclear protein, this interaction supports the idea that NQO1 is also in the nucleus (Winski, Koutalos et al. 2002). Taken as a whole, it seems that the degradation by the 20S proteasome of several short-life proteins such as p53, p63, p73, $p33^{ING1b}$ and ODC is the used pathway unless they are bound to a stabilizer like NQO1. It is known that p21 uses also the 20S proteasome pathway although the stabilizer agent in that case is PCNA (Touitou, Richardson et al. 2001). These results also show that one protein can undergo different degradation processes and suggest that NQO1 execute functions beyond its enzymatic action (Asher and Shaul 2005).

6. Apoptosis

6.1. An overview

Apoptosis, also called programmed cell death, produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells. Apoptosis has attracted great attention in recent years. After its discovery by Carl Vogt in 1842, apoptosis research was dormant for more than a century. Despite of not knowing the molecular bases, apoptosis and its implications in the development and function of different organs have been studied since XIX century (Peter, Heufelder et al. 1997, Kerr 2002, Clarke and Clarke 2012). Its rediscovery in the second half of the XX century, and the coining of the term apoptosis in 1972 by Kerr, Wyllie, and Currie, enriched this research field of science with new observations in several tissues (Kerr, Wyllie et al. 1972). The number of publications related to apoptosis has been growing exponentially every year ever since. The studies of J. Sulston, S. Brenner y H. R. Horvitz in Cambridge about cell lineages in *Caenorhabditis elegans* identified a number of apoptosis

regulating genes – the first evidence at that moment that cell death is an active process under genetic control. Many of these genes have mammalian homologs that, like their worm counterparts, regulate mammalian apoptosis. Apart from its role in metamorphosis in insects, these discoveries created a new interest about the cell fate and programmed cell death (Horvitz and Sulston 1980, Horvitz and Sulston 1990). In this nematode cell fate and programmed cell death caused the unchanging loss of 131 cells through the animal life. These investigators were awarded with a Nobel Prize of Physiology and Medicine in 2002. The elucidation of the signal transduction pathways of apoptosis has lead especially to the identification of specific death signaling molecules such as a new family of cysteine proteases, the caspases. Of note, it has also become clear that many diseases are characterized by deregulation of apoptotic programs (Peter, Heufelder et al. 1997).

The apoptotic process is active and is fine-tune regulated. The changes in the cell includes cell shrinkage, nuclear fragmentation, chromatin condensation and blebbing. Apoptosis produces cell fragments called apoptotic bodies that are phagocytized by immune system cells due to the externalization of phosphatidylserine to the plasma membrane external part (Majno and Joris 1995, Allen, Hunter et al. 1997).

Several decades of research have allowed to establish the main pathways of apoptosis activation. New findings elaborate a complex map of apoptosis-related interactions, being difficult to establish univocal pathways in the programmed cell death.

Traditionally, two apoptosis activation pathways have been described, the extrinsic and the intrinsic pathway. In the extrinsic pathway, plasma membrane receptors that belong to the superfamily of Tumor Necrosis Factor Receptor (TNFR), such as Fas, TRAIL-R1/2 or TNF-R are stimulated by extracellular ligand (TNF-α, FasL, TRAIL). The receptor death domains recruit adaptor proteins needed for the signal transduction of survival or death. In the case of Fas (CD95) and receptors for TRAIL, the Death-Inducing Signaling Complex (DISC) is created when adaptor proteins like Fas-associated protein with death domain (FADD) recruits through Death Effector Domain (DED) procaspase-8 and procaspase-10. Both procaspases are activated by proteolysis and then are able to activate the effector caspases, mainly caspase-3.

In the intrinsic pathway, intracellular stimuli can induce the activation of proapoptotic proteins that belong to the Bcl-2 family. The balance in the outer mitochondrial membrane between the proapoptotic members (Bax, Bak, Bid and others) and antiapoptotic (Bcl-2, Bcl-xL, Mcl-1 and others) of this family determine the pore formation and the subsequence

release to the cytosol of different proteins of the mitochondrial intermembrane space. Among these proteins we find the cytochrome *c*, required for the apoptosome formation together with Apaf-1 and procaspase-9. The apoptosome allows by ATP consumption the activation of caspase-9 and subsequently caspase-3 and other effector caspases (-6 and -7). Concurrently to the caspases-dependent pathway, there is a release to the cytosol from the intermembrane space of several factors that includes endonuclease G (EndoG) and Apoptosis Inducing Factor (AIF). These two factors perform a caspase-independent apoptosis when they translocate to the nucleus and degrade the genetic material (Figure 9). The ER apoptosis pathway is now being highly investigated (reviewed by (Gorman, Healy et al. 2012)). The ER is able to modulate or induce apoptosis by the Inhibitor Apoptosis Proteins (IAPs) or by the action of not very well characterized caspases (caspase-12) or caspase that seems to be involved in ER (caspase-8). The importance of apoptosis induced by ER stress is being studied in neurodegenerative, neoplastic and metabolic diseases. Other pathways able to regulate apoptosis have been described, including pathways involved in survival and growth. In addition, the ceramide production from sphingomyelin, catalyzed by several kinds of enzymes with sphingomyelinase activity can induce apoptosis by mechanisms that still need to be cleared (Wu, Clarke et al. 2010).

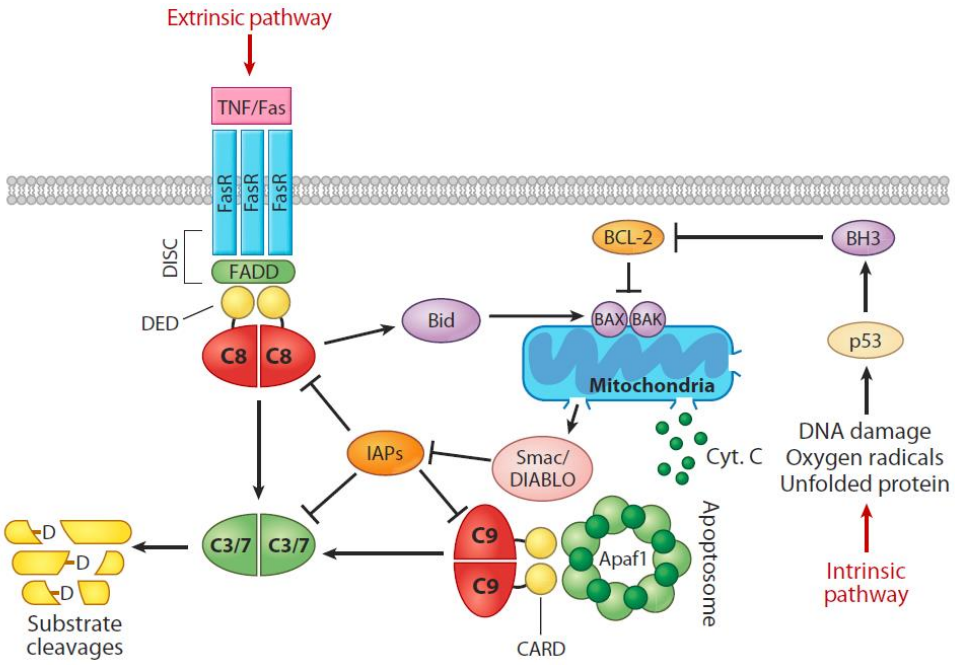

Figure 9: Basic outline of the extrinsic and intrinsic apoptosis pathways. In the extrinsic pathway, a ligand such as Fas ligand or TNF-α engages a cell membrane receptor, leading to formation of a death-inducing signaling complex (DISC). Caspase-8 (C8) is activated by association with the DISC and activate effector caspases-3 and -7 (C3/7) directly and/or by Bid cleavage, leading to mitochondrial permeabilization by Bax and Bak. In the intrinsic pathway, signals act through p53 and others to activate the BH3-only proteins, which

antagonize the antiapoptotic Bcl-2, leading to permeabilization of the mitochondria by Bax and Bak. Both pathways result in mitochondrial permeabilization, release of cytochrome *c* (Cyt. C) and apoptosome formation, being the latter the scaffold for activating caspase-9 (C9). Caspase-9 activates the effector caspases. Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI) is also released from mitochondria and antagonizes the IAP caspase inhibitors. Apaf1, apoptotic protease activating factor 1; Bak, Bcl-2 homologous antagonist/killer; Bax, apoptosis regulator BAX; Bid, BH3-interacting domain death agonist; CARD, caspase-recruitment domain. Figure taken from (Crawford and Wells 2011).

6.2. Caspases

Cysteine-aspartic proteases or caspases are a group of enzymes linked to a number of development and differentiation process. The most relevant among all their functions is the programmed cell death. Its enzymatic activity is based on a catalytic center able to adjust a tetrapeptide target that possesses an aspartic residue. More than 1000 different proteins, approximately the 5% of the cell total proteome, are susceptible of processing by a caspase (Crawford and Wells 2011).

Caspases are synthesized as zymogens, enzyme precursors that have a prodomain that should be removed by proteolysis in order to accomplish the active configuration. Frequently, the caspase activation happens by dimerization of the inactive form, cross-activation by proteolysis and configuration of an active tetramer (Khan and James 1998). Occasionally, a conformational change by association to other polypeptides that facilitates the processing or the breakup is catalyzed directly by other protease. The prodomain of caspases can contain recruiting domains (CARD) or DED that facilitate the location of some procaspases in the complexes where activation of some of them is carried out such as DISC or apoptosome.

In humans and rodents approximately a dozen of caspases have been described. They can be classified into three groups according to their specificity with the fourth residue of the target tetrapeptide (Thornberry, Rano et al. 1997):

1. Group I: Comprises the inflammatory caspases -1, -4 and -5 (-4 and -5 in mice correspond to caspase-11). They possess a long prodomain with CARD and higher affinity for the target tetrapeptides with a P4 of tryptophan or leucine.
2. Group II: Includes caspases with a short prodomain that interact with tetrapeptide with an aspartate in the P4 position. Effector caspases -3 and -7 belong to this group. Capase-3 is able to act on more than 300 substrates and caspase-7 on a hundred.
3. Group III: These caspases have a long prodomain and affinity for leucine or valine in the P4. This is the case of initiator human caspases -8 and -9 (both are also present in mice) and caspase-10.

Some members of the family cannot be classified distinctly as belonging to any of these groups. For instance caspase-2, with a homology with group I but affinity for aspartate in P4. Caspase-6 is an effector caspase with homology with group II, but affinity similar to group III (Crawford and Wells 2011).

Next we will study the initiator caspases -8 and -10, and also effector caspase-3. The caspase-9 will be described in the apoptosome section.

6.2.1. Caspases-8 and -10

Caspase-8 and -10 are classified as initiator caspases of the extrinsic apoptotic pathway and often are considered redundant in localization and action. Both of them can be found in human cells, but caspase-10 is not expressed in rodents. However, the relevance of caspase-10 is confirmed in humans since the caspase-10 deficiency is the origin of several proliferative diseases (reviewed by (Wachmann, Pop et al. 2010)). Caspase-8 belongs to group II as described above. Caspase-8 has a long prodomain containing DED which is able to interact with adaptor proteins of the complexes formed by receptors of extracellular ligands (Zhao, Sui et al. 2010). The role of the DED in the cytosol, once it dissociates from the active caspase, and also the role of caspase-8 when not bound to the DISC are still poorly understood. Procaspase-8 creates homodimers when recruited in the DISC. These homodimers possess enzymatic activity able to carry out proteolysis that generates heterotetramers with two long subunits (20kDa, p18) and two small units (12 kDa, p10). After several conformational changes and proteolysis processes, activated caspase-8 in the form of heterotetramer is released to the cytosol where acts on a number of targets. In the so-called type I cells, like lymphocytes, caspase-3 activation mediated by caspase-8 is enough to initiate the apoptotic process. On the other hand, in type II cells, like the hepatocytes, an amplification of the mitochondrial signaling through Bid is needed with the concomitant release to the cytosol of additional proapoptotic proteins from the intermembrane space (Walter, Schmich et al. 2008, Jost, Grabow et al. 2009). Also, the regulation of migration and adhesion by caspase-8 can explain normal or high levels of this caspase in several tumors (Barbero, Barila et al. 2008).

6.2.2. Caspase-3 and other effector caspases

Caspase-3 is the main apoptosis effector in the caspase-dependent pathway, where both extrinsic and intrinsic pathway converge. Caspase-3 is synthesized as a single-chain inactive zymogen, containing a prodomain, as well as large and small subunits that include the residues required for substrate recognition and cleavage. Caspase-3 activation occurs in

two stages. First, caspase-3 proforms are cleaved by upstream caspases, such as active caspase-8, at Asp175 to generate intermediate, yet still active, heterotetramer complexes consisting of two p19 and two p12 peptides (p19/p12 complexes). The second stage involves removal of the short prodomain from the p19 peptides by autocatalytic processing, and cleavage at residue Asp28, to generate the fully mature p17/p12 form of the enzyme (Han, Hendrickson et al. 1997). Several hundreds of caspase-3 targets have been identified, far above from other effector caspases (Crawford and Wells 2011), and studies *in silico* have determined that thousands of proteins are susceptible of being process by this caspase (Ayyash, Tamimi et al. 2012). One of the main target proteins of caspase-3 is a Caspase-activated DNase (CAD) which is responsible of the DNA break in the final stage of apoptotic process. In normal conditions, CAD is found in the cytosol, bound to ICAD, a repressor that acts as a chaperone. The processing of ICAD by caspase-3 allows the migration of CAD to the nucleus, where DNA undergoes breaks in the internucleosomal fragments. ICAD and CAD are also named DFFA and DFFN (DNA fragmentation factor) (Enari, Sakahira et al. 1998). CAD expression varies depending on the cell type. It is high in lymphoid organs suggesting a great importance of caspase-dependent apoptosis in the immune system, but its levels are reduced in liver or kidney and also in fibroblasts (Hail, Carter et al. 2006).

In a parallel way, caspase-3 suppresses the DNA reparation capacity of the cell through the poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is an abundant nuclear protein functioning as a DNA damage sensor enzyme. Poly(ADP-ribosylation) contributes to DNA repair and to the maintenance of genomic stability. On the other hand, oxidative stress-induces overactivation of PARP-1 that increases ATP consuming, culminating in cell dysfunction or necrosis. This cellular suicide mechanism has been involved in the pathomechanism of stroke, myocardial ischemia, diabetes, traumatic central nervous system injury, arthritis and various other forms of inflammation (Virag and Szabo 2002). The suggested mechanism of cell death is the enormous increase of the energy expenditure of the overactivation of PARP-1. Thus, processing of this enzyme by caspase-3 has been interpreted as a way of saving energy rather than a mechanism for suppressing DNA reparation. Caspase-3 can also affect some subunits of the proteasome, generally inhibiting its activity (Adrain, Creagh et al. 2004, Sun, Butterworth et al. 2004). However, in some cell types, such as muscle fiber, caspase-3 activity has been shown recently to increase the proteasome activity (Wang, Zhang et al. 2010).

Caspase-3 also acts on its own inhibitor, XIAP, establishing a positive feedback that increases the efficiency of the apoptotic process (Hornle, Peters et al. 2011). Many signaling and structural proteins, as well as proteins involved in metabolite transportation, survival

and other functions, are caspase-3 substrates (Fischer, Janicke et al. 2003). There is a similarity of function of caspase-3 with the rest of effector caspases, but there is not a complete redundancy. The caspase-3 and -7 double knockout mice die shortly after birth, but the knockout mice for either caspase have a relatively normal development (Lakhani, Masud et al. 2006). The relative importance of one protease depends on the apoptotic stimulus and the cell type (Lamkanfi and Kanneganti 2010). However, the proteolysis of PARP-1 and the DNA fragmentation are exclusive functions of caspase-3 and not caspase-7 (Lakhani, Masud et al. 2006). Caspase-6 is also an effector caspase according to its homology with caspase-3 and caspase-7, even though the differences with the target tetrapeptide affinity. The function of caspase-6 in the nuclear structure dismantling and its action on laminines (Orth, Chinnaiyan et al. 1996), which is required for the condensation of chromatin during the apoptotic process, are well known (Ruchaud, Korfali et al. 2002). Currently, the involvement of caspase-6 in neurodegenerative diseases such as Huntington or Alzheimer, and specifically its role in the axon degeneration, is gaining importance. (Graham, Ehrnhoefer et al. 2011).

6.3. Bcl-2 family of proteins

The main regulators of the mitochondrial apoptosis pathway are proteins that belong to the Bcl-2 family, a name given by the first member of this family that was discovered, the Bcl-2 polypeptide (B cell CLL/lymphoma 2).

They are cytosolic proteins or proteins integrated in the Outer Mitochondrial Membrane (OMM). In certain cases they can alternate both locations because of conformational changes. All of them share Bcl-2 homology domains (BH domains) in several combinations (reviewed by (Chipuk, Moldoveanu et al. 2010)). There are three groups of subfamilies of proteins in the Bcl-2 family:

1. Antiapoptotic proteins that contain four BH domains and are generally integrated within the OMM, although they may also be found in the cytosol or the endoplasmic reticulum. These proteins preserve the integrity of the OMM by directly inhibiting the proapoptotic members of the Bcl-2 family.
2. Proapoptotic effector proteins with a BH4 motif, like Bak (Bcl-2 antagonist killer) and Bax (Bcl-2 associated x protein)
3. The BH3-only proteins. Proteins that only contain the BH3 domain. They can be classified into two groups:

- Proteins that able to only interact with antiapoptotic members of the family. They are Bad, Puma, Bmf and Noxa. They are called derepressors and sensitizers.
- Proteins that interact with effectors and antiapoptotic proteins. They are Bid and Bim and are called direct activators.

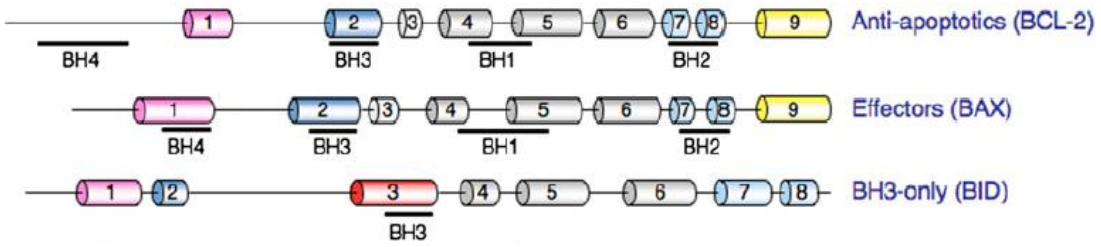

Figure 10: Bcl-2 Family Composition. The Bcl-2 proteins are comprised of Bcl-2 homology (BH) domains. A representation of an antiapoptotic (Bcl-2), effector (Bax), and BH3- only (Bid) protein is shown with the BH1-4 designated underneath the corresponding α-helices. Figure taken from (Chipuk, Moldoveanu et al. 2010).

6.3.1. Bcl-2

It was the first protein found in mammals with sequential and functional homology with Ced-9, an apoptosis regulator in *Caenorhabditis elegans* (Peter, Heufelder et al. 1997). Its role in lymphomas generation, through the suppression of the programmed cell death gave the name to the entire family (Vaux, Cory et al. 1988). Afterwards, deregulation of Bcl-2 levels was also observed in several type of tumors, and its role as antiapoptotic protein was elucidated.

Bcl-2 is found in the Outer Mitochondrial Membrane (OMM), where functions as a membrane permeabilization inhibitor. Bcl-2 is able to interact with proapoptotic effectors like Bax and Bak. Bcl-2 prevents the oligomerization of these effectors, thus impeding release of proteins from the intermembrane space (IMS) to the cytosol (Cheng, Wei et al. 2001, Green and Evan 2002). Bcl-2 KO mice have thymus and spleen regression and a polycystic renal disease among other phenotypes because of excessive apoptosis (Bouillet, Cory et al. 2001).

6.3.2. Bax

It is mainly a cytosolic protein that can be activated by other proteins – conformation modification by tBid, phosphorylation by Akt, ERK1/2, JNK, p38 or induction of its p53-mediated expression (reviewed by (Chipuk, Moldoveanu et al. 2010)) – as part of a cellular

response to extracellular stimuli or to an intracellular stress situation. It has been shown that in normal conditions a small fraction of cellular Bax is associated to the OMM and a constant transit of Bax between the OMM and the cytosol exists which is regulated by Bcl-xL and probably by other proteins of the Bcl-2 family (Edlich, Banerjee et al. 2011). The mechanism that regulates Bax migration has not been completely elucidated, but it is known that p53 (Chipuk, Kuwana et al. 2004), prostaglandins (Lalier, Cartron et al. 2011) or posttranslational modifications (Kutuk and Letai 2008) are involved. It is believed that the contact of Bax with the OMM could lead to conformational changes and that the membrane composition and mitochondrial morphology could determine the refractory or receptive properties toward Bax (Yethon, Epand et al. 2003, Tondera, Grandemange et al. 2009). From its activation by tBid, Bax is recruited to the OMM where is integrated and forms oligomers that make pores. There pores allow the release of IMS proteins like cytochrome *c* (Antonsson, Montessuit et al. 2000).

6.3.3. Bid

The direct activator Bid is responsible of the signaling transduction of cell death from extracellular ligands towards the proteins that regulate the permeability of the OMM as Bax and Bak (Luo, Budihardjo et al. 1998).

Bid is a cytosolic protein that undergoes proteolytic processing by caspase-8 to generate a 15kDa fragment known as truncated Bid (tBid). This fragment interacts with Bax, which is thus activated and its integration into the OMM facilitated. This is the first step for the pore creation in the mitochondria. It has been proposed that tBid integration in the OMM is needed for Bax recruitment and activation (Lovell, Billen et al. 2008). The affinity of tBid for the mitochondria is determined by the presence of a central α-helix able to bind to cardiolipins (Kuwana, Mackey et al. 2002). Also, the mitochondrial carrier homologue 2/Met-induced mitochondrial protein (MTCH2/MIMP) has been identified as a major regulator of tBid recruitment. The presence of this protein increases the efficiency of the capture of tBid (Zaltsman, Shachnai et al. 2010). Bid is also able to interact with antiapoptotic proteins like Bcl-2 and Bcl-xL. It is believed that, by binding to these proapoptotic proteins, Bid acts as a repressor (Martinou and Youle 2011).

6.3.4. Other members of the Bcl-2 family

The role of different members of the Bcl-2 family has been studied mainly in relation with the processes of development and cancer. The map of interactions for this family of proteins is wide and cross several signaling pathways related with growth factors, intracellular

damage sensors, cytokines and extracellular ligands. BH3-only proteins Bad, Bim, Bik, Bmf, Hek, Nix, Noxa and Puma are regulated by DNA damage, endoplasmic reticulum stress and heat shock, lack of growth factors, absence or presence of certain cytokines and other stimuli (Chipuk, Moldoveanu et al. 2010).

Different from Bax, and despite of its functional homology, the proapoptotic protein Bak is located in the OMM. The activation of this protein by the binding of a BH3-only direct activator causes conformational changes that result in the oligomerization of Bak (Dewson, Kratina et al. 2008).

Some of the antiapoptotic proteins like Bcl-xL or Mcl-1 – that were found later than Bcl-2 – have shown a more drastic phenotype when absent. Knockout mice for any of this proteins die in the embryonic stage. Mcl-1 knockout mice die because of the lack of implantation of the embryo at blastula stage (Rinkenberger, Horning et al. 2000). In the case of the Bcl-xL knockout animals, cell death is due to massive apoptosis of neurons and hematopoietic cells (Motoyama, Wang et al. 1995).

6.4. Permeabilization of the OMM

Mitochondria is the organelle in charge of the generation of molecules with high energy bounds: ATP. The production of ATP is possible because of the mitochondria compartmentalization. These compartments include the OMM; the IMS, the inner mitochondrial membrane (IMM) and the matrix. The proton gradient in the IMS in relation to the matrix, due to the electron transport chain, allows for the functioning of the ATP synthase and ATP generation.

However, apart from being a source of chemical energy, mitochondria is also involved in cell signaling. The IMS is also the reservoir of several molecules with a key role in the apoptosis intrinsic pathway and also the caspase-independent pathway. The release of these proteins to the cytosol constitutes the point of no return of the cell death process (Garrido, Galluzzi et al. 2006). Cytochrome *c*, in addition to its role in the oxidative phosphorylation, is part of the apoptosome together with Apaf-1 and procaspase-9 (Bratton and Salvesen 2010). The Smac/Diablo protein blocks the action of apoptosis inhibitors (IAPs), while AIF and EndoG cooperate to induce the caspase-independent apoptosis.

The event that promotes the release of the already mentioned proteins to the cytosol is the Outer Mitochondrial Membrane Permeabilization (OMMP). The molecular basis of this process has not been completely elucidated. Two models have been suggested to explain

the release of proteins from the IMS to the cytosol and they are not mutually exclusive (Kinnally and Antonsson 2007). On one hand, mitochondrial permeability transition (MPT) through the permeability transition pore complex (PTPC) formation allows the release of matrix and IMS proteins to the cytosol. On the other hand, the mitochondrial apoptosis-induced channel (MAC), generated by the interaction of proteins of the Bcl-2 family, mediates the release of proteins from the IMS.

6.4.1. The Permeability Transition Pore complex (PTPC)

The PTPC is a multiprotein complex containing Voltage dependent anion channels (VDAC), located in the OMM; the adenine nucleotide transporter (ANT) in the IMM, the cyclophilin D of the matrix and others (Green and Kroemer 2004). Its composition is still a matter of debate as it has been shown than the ANT is not an essential component (Kokoszka, Waymire et al. 2004). The opening of the PTPC triggers the depolarization of the IMM and an osmotic increase of the mitochondria matrix volume. Given the higher surface of the IMM that form the cristae, the increase of the volume causes a rupture of the OMM and the release of IMS proteins to the cytosol (Kinnally and Antonsson 2007). However, it has been demonstrated that the release of cytochrome *c* (at least partially) can exist without altering the membrane integrity and triggering depolarization (Guo, Pietkiewicz et al. 2004, Dejean, Martinez-Caballero et al. 2005). In accordance with studies carried out with cyclophilin D-deficient mice, this mechanism for OMM rupture could be involved in cellular necrosis but not apoptosis (Nakagawa, Shimizu et al. 2005).

6.4.2. Mitochondria Apoptosis-induced Channel (MAC)

The alternative to the PTPC is the MAC. This structure is consisted of multiple units of Bax and Bak that interact to allow the release of proteins to the cytosol. Regulation of the MAC seems to be dependent on the Bcl-2 family, although it is believed that other components might be also involved (Antonsson, Montessuit et al. 2001, Dejean, Martinez-Caballero et al. 2006). This channel is created without interference of the IMM. Several Bcl-2 family proteins are similar to the bacterial toxins that are able to generate pores in the cell membranes (Petros, Olejniczak et al. 2004). Bcl-2, Bax, Bak and Bcl-xL are able to generate pores in artificial lipid membranes, but some experiments dismiss this idea under physiologic conditions (Murphy, Schneider et al. 2001, Dejean, Martinez-Caballero et al. 2006).

However, Bax can create oligomers that make voltage independent channels. These channels allow the release to the cytosol of molecules up to 5nm (Roucou, Rostovtseva et al.

2002). A number of studies confirmed that Bax constitute an essential component of the MAC (reviewed by (Dejean, Martinez-Caballero et al. 2006)). Because of the functional and sequence homology with the protein Bak, both proteins are considered redundant on its role in apoptosis regulation and the silencing of both of them is needed in order to produce resistance to a variety of cell death stimuli (Wei, Zong et al. 2001). The release of cytochrome *c* to the cytosol takes place in an earlier stage compared with to the release of the rest of proteins from the IMS, with higher molecular weight, that requires caspase activation (Adrain, Creagh et al. 2001, Arnoult, Gaume et al. 2003). A gradual increase of the pore diameter along with the apoptotic process has been suggested. In other cases, the release is however almost simultaneous (Stoica, Movsesyan et al. 2005, Munoz-Pinedo, Guio-Carrion et al. 2006).

A combination between Bax and Bid can form pores that allow the release of molecules up to 2 MDa in preparations of OMM *in vitro*. This demonstrates that other proteins besides the Bcl-2 family of proteins are not needed to release proteins from the IMS to the cytosol (Kuwana, Mackey et al. 2002). This activity requires the presence of cardiolipins in the outer layer of the OMM. The presence of cardiolipins could be an additional regulator factor since these lipids are present at low levels at this location. The number of Bax monomers participating in the OMMP and its *in vivo* relevance are still under discussion, but a number of several hundred has been estimated. Both components of PTPC as well as the Bcl-2 family of proteins seem to accumulate around the contact area between IMM and OMM. The actual physiological functioning of the OMMP still remains to be cleared, but seems to exist a cooperation between these two systems, and one or another could prevail depending of the apoptotic stimulus and the cell type (Zamzami and Kroemer 2001, Scorrano, Ashiya et al. 2002). Despite these evidences, some researchers (Tait and Green 2010, Westphal, Dewson et al. 2011) have minimized the importance of PTPC in the apoptotic process.

6.4.3. Biphasic release of cytochrome *c*

The release of cytochrome *c* to the cytosol takes place in two phases. At first, the OMMP allows the exit of cytochrome *c* molecules that are either free in the IMS or bound in a lax way to the IMM. This release does not induce cell death. Subsequently, because of an amplification mechanism, there is an exit of a second wave of cytochrome *c*. The involved mechanisms are the oxidation of cardiolipins by ROS or the proteolytic action of caspase-2 and -3 (Garrido, Galluzzi et al. 2006).

6.5. The apoptosome

The exit of the cytochrome *c* to the cytosol induce the apoptosome complex formation. This complex is very different to the DISC but has also the capacity of bringing together initiator procaspases, where the activation is facilitated and the apoptotic signal transmitted. The apoptosome complex is consisted of cytochrome *c*, Apaf-1 and caspase-9 (Bratton and Salvesen 2010).

6.5.1. Cytochrome *c*

Cytochrome *c* is an IMM peripheral protein and is in charge of the electron transport between complex III and IV of the electron transport chain. It is synthesized in the cytosol as a 104 amino acid chain. It has a 3.4 nm diameter and is translocated to the IMS and then combined with a heme group. The electron transport and the apoptosome formation involve different protein domains. This makes possible the generation of mice with active oxidative phosphorylation but exhibiting a phenotype similar to knockouts in caspase-9 and Apaf-1 (Hao, Duncan et al. 2005).

6.5.2. Apaf-1

Apoptotic protease activating factor 1 (Apaf-1) is a multidomain protein that contains caspase recruitment domains in the N-terminus, a nucleotide bound and oligomerization domain (NOD) and a WDR C-terminus domain that contains a dozen of WD40 repetitions, a β-helix domain to facilitate protein-protein interactions (Bratton and Salvesen 2010).

The binding of cytochrome *c* to the WDR domain and of ATP to the NOD cause conformational changes in Apaf-1 which are dependent on ATP hydrolysis, to allow for the generation of a heptamer. In this heptamer the CARD are exposed in order to be recognized by the procaspase-9 units (Yuan, Yu et al. 2010).

6.5.3. Caspase-9

Caspase-9 is a protease with an important role in apoptosis. Caspase-9 activates the effector caspase-3 as a consequence of the intrinsic pathway signaling. Dimerization and cross-activation is required. The main role of the apoptosome is the agglutination of the procaspase-9, increasing the local concentration of the molecules in order to facilitate activation (Bratton, Walker et al. 2001). Caspase-9 has some differences when compared to the rest of initiator caspases. Firstly, caspase-9 is only active while bound to the multimeric complex. Second, the presence of an activation loop. The amino acidic sequence that links the large and the small subunits is larger than in caspase-8 and -10 and permit the access to

the active site without requiring a proteolytic rupture. Despite procaspase-9 is active since the binding with Apaf-1, proteolysis always happens. It has been suggested that the processing of procaspase-9 has the purpose of establishing a "molecular clock" that defines the duration of the apoptosome activity depending on the amount of procaspase-9 in the cytosol. Procaspase-9 molecules have more affinity for the apoptosome than caspase-9. This allows the substitution of the cleaved molecules, because they are no longer active once they dissociate from Apaf-1 (Malladi, Challa-Malladi et al. 2009).

6.6. Caspase-independent apoptosis

Several IMS proteins (AIF and EndoG) are able to initiate the degradation of the genetic material of the cell without the participation of caspases. This action takes place once they are released to the cytosol and translocated to the nucleus, even under conditions where caspase-dependent pathways may be inactivated (Munoz-Pinedo, Guio-Carrion et al. 2006).

6.6.1. Apoptosis-inducing factor (AIF)

AIF is a 67 KDa transmembrane flavoprotein of the IMM (Otera, Ohsakaya et al. 2005). Its mitochondrial functions are not completely elucidated but it is apparently involved in the redox balance of the mitochondria because of its NADH-oxidase capacity (Miramar, Costantini et al. 2001). A role for AIF as a stabilizer of the complex I has been suggested, but it is still unclear whether or not AIF plays a direct role in the electron transport chain or it is only an antioxidant enzyme (Joza, Pospisilik et al. 2009). After the apoptotic stimulus and the permeabilization of the OMM, a protease (probably a calpain or a cathepsin) splits the AIF transmembrane domain, releasing the cleaved protein and allowing its exit from the IMS to the cytosol (Polster, Basanez et al. 2005).

Once released, AIF can have more than one destination, being its role in the nucleus the most studied. AIF translocates after its association with cyclophilin A, a protein that is considered essential for the AIF apoptotic function (Cande, Vahsen et al. 2004). According to the orthologous genes in *Caenorhabditis elegans*, once AIF is translocated to the nucleus (with no endonuclease activity *per se*), interacts with EndoG. This interaction confers the nuclease activity and activates the degradation of DNA (Wang, Yang et al. 2002). AIF performs other functions in different compartments of the cell. For instance, at the plasma membrane AIF is able to recruit a flippase-like protein named SCRM-1 (because of the phospholipid-scrambling activity from the inner layer of the lipid bilayer to the outer layer to promote phosphatidylserine externalization) (Susin, Lorenzo et al. 1999, Wang, Wang et al. 2007), which constitutes a stimulatory signaling for phagocytosis, the final destiny of apoptotic cells. AIF also binds to a subunit G of the eukaryotic translation initiation factor 3 (eIF3g)

and stops the synthesis of new proteins during the apoptotic process. There is disagreement about the mechanism of AIF release. Some groups have suggested that the activity of a caspase might be needed to promote the release of AIF and EndoG from the IMS to the cytosol, a requirement that is not needed in the case of cytochrome *c*, Smac/Diablo and HtrA2/Omi (Arnoult, Gaume et al. 2003). However, other research groups have not been able to reproduce this fact in other models (Susin, Lorenzo et al. 1999, Daugas, Susin et al. 2000). PARP-1 is also able to activate a caspase-independent apoptosis through the release of AIF, in addition to the function in the caspase-3 regulation (Cregan, Dawson et al. 2004).

6.6.2. Endonuclease G (EndoG)

Endonuclease G (EndoG) is a mitochondrial nuclease of 30 KDa located in the IMS that it seems to be bound to the IMM (Varecha, Amrichova et al. 2007). It is also released to the cytosol after the permeabilization of the OMM, and its translocation to the nucleus allows the fragmentation of the genetic material in a caspase-independent way (Li, Luo et al. 2001). EndoG acts in an unspecific way on any type of nucleic acid, preferentially single strand DNA. Even in the case of double strand DNA, EndoG carries out breaks in only one strand (Widlak, Li et al. 2001). The physiological meaning of EndoG action in each cell type is not well known. Indeed, some groups have reported no phenotypic changes in the EndoG knockout mice (Irvine, Adachi et al. 2005, David, Sasaki et al. 2006), while an earlier studied showed the requirement of this enzyme for the embryonic development (Zhang, Dong et al. 2003). EndoG may participate in processes like ischemia damage (Nielsen, Lambertsen et al. 2009) or muscle atrophy (Leeuwenburgh, Gurley et al. 2005, Marzetti, Wohlgemuth et al. 2008). EndoG could partially substitute the CAD in hepatocytes and neurons, in which there is a high quantity of mitochondria and a reduced expression of CAD (Hail, Carter et al. 2006). EngoG is found in rat and mouse hepatocytes and hepatoma cell lines (Bajt, Cover et al. 2006, Ishihara and Shimamoto 2006). However, a tendency to lose EndoG expression has been reported in human hepatocellular carcinoma (Ahn, Jeong et al. 2008).

6.6.3. Other proteins that regulate apoptosis

6.6.3.1. Inhibitor of Apoptosis Proteins (IAPs)

This family of proteins is well-conserved in many organisms, from yeasts to mammals, as well as in viruses. They contain between one and three copies of a domain called Baculovirus IAP Repeat (BIR). The nomenclature BIRC1-8 (BIR-containing protein) is used for the members of this family identified in humans (Dubrez-Daloz, Dupoux et al. 2008). These proteins are able to interact with apoptosis-related proteases, but the role of many of them is still not clear. The best characterized and studied protein is the X-linked inhibitor

of Apoptosis (XIAP) which is an important repressor of caspase-dependent apoptosis (Holcik and Korneluk 2001, Dubrez-Daloz, Dupoux et al. 2008). XIAP contains three BIR domains and one Zinc finger domain called RING. The function of the BIR domains is the interaction with IAP binding motif (IBM). The IBM can be found in proteins such as Smac/Diablo and HtrA2/Omi and caspase-3, -7 and -9. The RING domain acts as a ligase of the ubiquitine E3 and can mediate its own ubiquitylation and the ubiquitylation of the target proteins (Dubrez-Daloz, Dupoux et al. 2008).

Effects of XIAP deletion in mice are not as deleterious as those observed in *Drosophila melanogaster*. In the latter, early embryonic death is registered (Wang, Hawkins et al. 1999, Harlin, Reffey et al. 2001). The 90% of hepatocellular carcinomas (HCC) have an increase of XIAP which correlates with metastasis and tumor recurrence (Shi, Ding et al. 2008). Also, it regulates survival of neurons (Potts, Singh et al. 2003) and cardiomyocytes (Potts, Vaughn et al. 2005).

XIAP can be processed by caspase-3 and -7. The generated fragments maintain the inhibitory capacity but a different specificity. One of the fragments (containing BIR3 and RING) is able to inhibit the effector caspases whereas the other (with BIR1 and BIR2) has the inhibitory capacity on caspase-9 (Deveraux, Leo et al. 1999).

Philipp Jost and others (Jost, Grabow et al. 2009) described a mechanism that defines two type of cells in relation to its cell death pathways: those in which extracellular stimuli are enough to trigger apoptosis by themselves (type I) like lymphocytes, and those in which Bid processing and signal amplification of the mitochondrial pathway is needed for the cell death (type II). Inhibition of caspase-3 by XIAP can impede apoptosis from caspase-8 signaling. However, the release of IMS factors, some of them able to inhibit Xiap, like Smac/Diablo could allow the activation of effector caspases.

6.6.3.2. Smac/Diablo

This protein is known as second mitochondria-derived activator of caspases or direct IAP binding protein with low pI (Smac/Diablo). Smac/Diablo is encoded by nuclear genes but located the mitochondrial IMS. The permeabilization of the OMM in response to an apoptotic stimulus allows its exit to the cytosol together with the rest of the IMS proteins (Du, Fang et al. 2000). Once in the cytosol, Smac/Diablo dimerizes to inhibit Xiap through the interaction of its BIR2 and BIR3 domains (that interact with caspase-3 and -9, respectively). Smac/Diablo allows for a complete activation of the caspase cascade (Chai, Du et al. 2000). There is a decrease in the expression of Smac/Diablo in several tumors and

an inverse correlation of the level of this protein with the capacity of tumor progression (Martinez-Ruiz, Maldonado et al. 2008).

6.6.3.3. HtrA2/Omi

The HtrA2 (high-temperature requirement), also known as Omi, is one of the four members of the family Htr that is found in mice and humans. HtrA2/Omi is the only protein among the four that is found in the mitochondrial IMS, although it can also appear in the nucleus (Gray, Ward et al. 2000, Vande Walle, Lamkanfi et al. 2008). HtrA2/Omi is a 49 kDa protein synthesized in the cytosol and then translocated to the mitochondria because of the presence of a signal peptide. Once in the IMS, HtrA2/Omi undergoes proteolysis and generates a mature protein that have lost 133 amino acids. In the N-terminus HtrA2/Omi exposes an IBM domain to bind IAPs. The release of proteins from the IMS allow that this domain binds to XIAP and activates, in an indirect way, caspase-3, -7 and -9.

The exact function of HtrA2/Omi in the mitochondria is not clear, but the defect of this protein correlates with a higher incidence of neurodegenerative diseases like Parkinson (Strauss, Martins et al. 2005).

6.6.3.4. Hsp70 and other Heat Shock Proteins

After a variety of chemical and physiological stimuli, cells are able to rapidly induce the heat shock proteins (Hsp), highly conserved in evolution (Arya, Mallik et al. 2007). Many studies have been published about the function of these proteins as modulators of different apoptosis pathways (Samali and Orrenius 1998, Garrido, Gurbuxani et al. 2001, Beere 2005, Arya, Mallik et al. 2007). The Hsp, with inducible or constitutive expression, are classified according to their molecular weight in a series of families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp27 and Hsp10. The common feature among them is the molecular chaperone function. Their basic function is the correct folding of the synthetized polypeptides (and correction, if possible), the regulation of protein degradation and the translocation of proteins to subcellular locations as indicated by their corresponding signal peptides (Hartl and Hayer-Hartl 2002).

Hsp70 is the most studied member of the Hsp70 family. This protein attenuates the apoptotic signaling at several levels. It inhibits Bax translocation to the mitochondria (Gotoh, Terada et al. 2004, Stankiewicz, Lachapelle et al. 2005) and also the activation of Bid (Gabai, Mabuchi et al. 2002), reducing the release of cytochrome *c* to the cytosol (Mosser, Caron et al. 2000). Hsp70 also inhibits the apoptosome formation through the interaction with Apaf-1 (Beere, Wolf et al. 2000, Saleh, Srinivasula et al. 2000). However, it is not clear

whether it interferes with the oligomerization of Apaf-1 or it causes an incompatible conformation of the oligomer that is not able to bind caspase-9. Hsp70 inhibits the stress kinase cascade (Beere 2004, Lee, Lee et al. 2005) as well as the function of p53 (Wadhwa, Yaguchi et al. 2002). Recently the importance of Hsp70 in the inhibition of apoptosis for tumor development has been studied (Yang and Li 2009). The overexpression of Hsp70 is enough to induce lymphoma in mice (Seo, Park et al. 1996) and its silencing sensitizes cells to apoptosis in different tumor models (Nylandsted, Rohde et al. 2000, Gurbuxani, Bruey et al. 2001). High levels of Hsp70 involve a bad prognosis in human neoplasias (Sherman and Multhoff 2007), probably because of its antiapoptotic functions inhibiting part of the apoptotic machinery (Garrido, Schmitt et al. 2003).

Hsp70 has also a role in the inhibition of the caspase-independent apoptosis pathway. After AIF release to the cytosol, Hsp70 is able to sequester this protein impeding its translocation to the nucleus and the execution of its function as an apoptosis effector (Gurbuxani, Schmitt et al. 2003). The silencing of Hsp70 is enough to sensitize HepG2 cells to chemotherapeutic drugs and to increase the apoptosis levels induced by AIF (Yang and Li 2009)

Objectives

The main objective of this work is to analyze the effects of the lack of the transcription factor Nrf2 or its downstream target, the antioxidant enzyme NQO1, on growth, apoptotic signaling and proliferation pathways in MEFs. For this purpose, several apoptotic and proliferative markers will be analyzed and some functional characterizations will be also performed.

- Determination of alterations in the apoptotic signaling pathway of wild-type, Nrf2KO and NQO1KO MEFs. This partial objective will be developed through the study of different apoptotic markers, which will be compared among the three genotypes. Functional experiments using inhibitors will be also performed in order to elucidate the apoptotic pathways that are involved in the apoptosis process in cells lacking Nrf2 or NQO1.
- Determination of proliferation capacity. Proliferation assays will be performed as well as measurements of different proliferation markers.
- Study of the mitochondrial ultrastructure and mitochondrial dynamics in wild-type, Nrf2KO and NQO1KO MEFs. Ultrastructure will be studied by electron microscopy. Fussion and fision markers will be analyzed and compared among genotypes.

Materials and Methods

1. Murine Embryonic Fibroblasts (MEFs)

Wild-type (Wt), Nrf2KO and NQO1KO mice were maintained at the Gerontology Research Center, National Institutes of Health (Baltimore, MD).

1.1. MEFs preparation

MEFs were obtained from fetuses of the three genotypes at day 13 post-coitum. Pregnant female mice were sacrificed by cervical dislocation. The uterus was dissected and washed in Hank´s saline solution (Sigma, Spain). Each embryo was separated from its placenta and surrounding membranes. Head and dark red organs were also removed. After washing, embryos were finely minced and the resulting cells washed in Hank's saline solution and then resuspended in 1 ml of 0.25% Trypsin-EDTA (Sigma, Spain) per embryo for 15 minutes at 37°C. Trypsin was neutralized with complete DMEM. Non disaggregated tissue was eliminated, and the cellular suspension washed with 2 volumes of fresh culture medium. After centrifugation, the cell pellet was suspended in Dulbecco´s modified essential medium (DMEM) (Lonza, Spain) supplemented with 10% fetal bovine serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B, 2 mM L-glutamine, and 0.2% glucose (MEFs medium) and cells from each embryo were plated in 10 cm diameter dishes (passage 0). The medium was changed after 24 hours, being fibroblasts the only cells able to adhere to the culture surface. Cellular confluence was obtained after few days. Cells were then frozen and maintained under liquid nitrogen until used for the different determinations.

For cryopreservation, cells were frozen in 10 % dimethylsulfoxyde (DMSO) (Carlo Erba, Spain) prepared in complete media solution in cryogenic vials. Vials were placed overnight into an isopropanol freezing container placed in a -80°C deep freezer. Under these conditions, the cells freeze at 1°C per minute in the isopropanol freezing container. The following day, the frozen vials were quickly transferred to a liquid nitrogen storage tank using metal forceps.

For thawing cells, cryogenic vials were immersed into a 37°C water bath until no ice crystals were observed. Cells were then washed once with complete media in order to remove any DMSO prior to plating in culture flaks.

1.2. Generation of immortalized cell lines

After thawing, cells were cultured in MEFs medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Passages of cell cultures were carried out according to the procedure for obtaining the 3T3 cell line (Todaro and Green 1963). Primary fibroblasts were cultured in 75 cm^2 bottles at 4000 viable cells/cm^2. Passages were performed each 3 days, avoiding cell confluence. After each passage, cells were detached from culture plates using a Trypsin-EDTA detaching solution.

1.3. Viability assays

1.3.1. Trypan blue-exclusion assay

The number of viable cells were counted with a haemocytometer (Scharlab, Spain) by trypan blue-exclusion assay, a test used to determine the number of viable cells present in a cell suspension. The test is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells are unable to exclude the dyes (Strober 2001). In this test, viable cells exhibit a clear cytoplasm whereas nonviable cells are stained blue. Cell suspensions were mixed with trypan blue solution (Sigma, Spain) and then visually examined at the inverted microscope to determine whether cells take up or exclude dye. This method was followed for routine maintenance of cell cultures.

1.3.2. MTT assay

Viable cells with active metabolism convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) into a purple colored formazan product with an absorbance maximum near 570 nm, whereas nonviable cells lose the ability to convert MTT into formazan. Thus, color formation serves as a useful and convenient marker of viability (Riss, Moravec et al. 2004). To perform MTT viability assay cells were washed once with Hank's solution and incubated in MTT (Sigma) at 0.5mg/ml in DMEM (supplemented with 10% bovine serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B, 2 mM L-glutamine, and 0.2% glucose) without phenol red for three hours at 37°C. After incubation, the MTT working solution was removed. The converted dye was solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol). The extracted dye was then transferred to disposable plastic cuvettes and the absorbance measured at 570 nm in a spectrophotometer DU-640 (Beckman Coulter).

1.4. Thymidine incorporation assay

This cell proliferation assay measures the rate of incorporation of the radiolabeled DNA precursor [methyl-^{3}H]-thymidine into the replication strands of DNA produced during S

phase of the cell division cycle. Cells were incubated with 0.25 μCi/ml [methyl-^{3}H] thymidine (Amersham Pharmacia Biotech) for 24 hours. Then, wells were washed with 0.9% NaCl in order to remove any non-incorporated radioprobe. Cells were incubated with 5% trichloroacetic acid (TCA) (Merck) for 30 minutes at 4°C. Subsequently, cells were lysed with 0.1N NaOH (Panreac) for 90 minutes while shaken at room temperature. One aliquot (400 μl) of the lysates was added to 4 ml of scintillation cocktail (Cocktail Biogeen 1, Scharlau) and the quantity of [methyl-^{3}H] thymidine incorporated in the DNA was measured on a scintillation beta-counter Beckman LS 6000TA. Counts were normalized and referred to the quantity of protein (cpm/μg protein) using Bradford assay (Bradford 1976).

1.5. Wound healing assay

The wound healing assay assesses the migration capacity of cells. Cells were grown to a 90% of confluence. At this point a line was drawn with a marker on the bottom of the well. Using a sterilized 200 μl tip we scratched several wounds moving perpendicular to this line. The cells were rinsed very gently to remove any debris and fresh media was then added. Pictures were taken at a magnification of 10X just above and below the drawn line. After 8 hours pictures were taken again in the same area. The surface of the area without cells at time zero and after 8 hours was measured and the healing process quantified by ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

1.6. Flow cytometry

Samples were analyzed is an EPICS XL flow cytometer (Beckmann Coulter) equipped with a 488nm laser and the following filters: a 488nm dichroic long pass filter, a 488nm blocking filter, a 550nm dichroic long pass filter and a 525nm band pass filter for the first fluorescence channel (FL1), a 600nm dichroic long pass filter and a 575nm band pass filter for the second fluorescence channel (FL2), a 645nm long pass filter and a 620nm band pass filter for the third fluorescence channel (FL3), and a 675nm band pass filter for the fourth fluorescence channel (FL4).

1.6.1. Measurements of ROS levels in cells

The cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) was used to measure the levels of intracellular peroxides. In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH), which can be rapidly oxidized to highly fluorescent 2', 7'- Dichlorodihydrofluorescein (DCF) by intracellular peroxides. The fluorescence intensity is thus proportional to the ROS levels within the cell.

The probe (obtained from Merck) was freshly prepared in DMSO and added to the cells at a final concentration of 10μg/ml. Cells were incubated with the probe for 20 minutes at 37°C. After the incubation, cells were washed with Hank's saline solution to eliminate the excess of probe and then detached from culture plates using a Trypsin-EDTA solution. Cell pellets were washed twice with Hank's saline solution and resuspended in Hank's saline solution for peroxides measurements. After excitation at 488 nm, the fluorescence emission was registered at 525 nm (FL1).

The data were obtained in a logarithmic scale and were represented in histograms using the EXPO32 ADC Analysis software (Beckman-Coulter). For the quantification of the results, median data were used as those values are the most appropriate when a logarithmic scale is used (Shapiro 2003).

1.6.2. Measurements of Mitochondrial Membrane Potential (ΔΨ)

For measuring mitochondrial membrane potential we used the lipophilic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1 probe). JC-1 stands for 1st J-aggregate-forming cationic dye. The fluorochrome can be found as a monomer or as an aggregate. The monomer emits at 527 nm after excitation at 490 nm. Depending on the membrane potential, JC-1 is able of forming aggregates that are associated with a large shift in emission (590 nm). The color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized (Cossarizza, Baccarani-Contri et al. 1993). JC-1 forms monomers at low dye concentrations, and aggregates at higher concentrations. Both JC-1 aggregates and monomers exhibit fluorescence in the green end of the spectrum which is measured in the Green (FL-1) channel on the flow cytometer. When live cells are incubated with JC-1, the probe penetrates the plasma membrane of cells as monomers. Uptake of JC-1 into mitochondria is driven by the Δψ. The Δψ of healthy mitochondria is polarized and JC-1 is rapidly taken up by such polarized mitochondria. This uptake increases the concentration gradient of JC-1 leading to the formation of JC-1 aggregates within the mitochondria. JC-1 aggregates show a red spectral shift resulting in higher levels of red fluorescence emission which is measured in the Red (FL-2) channel. JC-1 does not accumulate in mitochondria with depolarized Δψ and remains in the cytoplasm as monomers. Depolarized Δψ indicates altered mitochondrial function which may be due to apoptosis or other cellular processes. For this assessment we used the Flow Cytometry Mitochondrial Membrane Potential Detection Kit of BD Bioscience according to manufacturer's instructions. The data were obtained are the mean of the

FL2/FL1 ratio and were represented in histograms using the EXPO32 ADC Analysis software (Beckman-Coulter).

1.7. Microscopy

1.7.1. Electron microscopy analysis

Cells were prefixed by adding glutaraldehyde to the cell culture for 5 minutes to a final concentration of 2.5%. Thereafter cells were scrapped and transferred to a vial, centrifuged and the supernatant discarded. After the prefixation, the fixation was carried out with 2% paraformaldehyde-2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH7.2 for 10 minutes at room temperature and subsequently for 2 hours at 4°C. The postfixation was in 1% osmium tetroxide in the same buffer for 1 hour at 4°C. After dehydration in an ascendant series of ethanol (50%, 70%, 90% and 100%), samples were transferred to propylene oxide and sequentially infiltrated in EMbed 812 resin. We used the sequence propylene oxide-resin 1:1, and 1:2 overnight. Afterwards samples were transferred to pure resin for 24 hour and then allowed to polymerize for 48 hours at 65°C. After sculpting, blocks were sectioned in an Ultracut Reicher ultramicrotome to get ultrathin (about 50nm width) sections. The sections were stained in aqueous 2% uranyl acetate for 5 minutes and Reynold's lead citrate for 5-8 minutes. The sections were viewed and photographed in a JEOL JEM 1400 transmission electron microscope.

Planimetric analysis of mitochondria was performed using the software ImageJ. Using the planimetric data we calculated mitochondrial area, circularity, number of mitochondria per μm^2 of cytoplasm and portion of cytoplasm occupied by mitochondria.

1.7.2. Phosphorylated histone H2A.X (DNA damage response)

Accumulation of spontaneous DNA lesions in MEFs of the different genotypes was investigated by immunostaining for phosphorylated histone H2A.X (γH2A.X), a marker of DNA double-strand breaks (DSBs). The intensity of the H2A.X staining was studied by confocal microscopy. For this purpose, cells grown on coverslips were fixed in 4% paraformaldehyde (PFA) in 0.01M PBS pH7.4 for 10 minutes at room temperature. Next, cells were washed twice with phosphate buffer saline (PBS) (sigma) and subsequently incubated with blocking solution (1% BSA, 0.3% Triton X-100 in PBS) for one hour. Then, cells were incubated overnight at 4°C with the primary antibody anti-phosphorylated H2A.X (Ser 139) (Cell Signaling) diluted in blocking solution. The antibody was removed by washing three times with PBS and cells were incubated with the secondary antibody conjugated with Alexa fluor 488 (Molecular Probes, Eugene, OR, USA) at room temperature

for two hours. Cells were washed three times with PBS. The coverslips were mounted on a slide with a drop of mounting medium containing the nuclear fluorescent dye DAPI [0.1M Tris-HCl, 70% glycerol, 2% N- propylgallate, 1 µg/ml DAPI (Sigma), pH 9]. DAPI (4',6-diamidino-2-phenylindole) is a DNA-specific probe which forms a fluorescent complex by binding the minor grove of A-T rich sequences of DNA (Kapuscinski 1995).

Preparations were observed in a LSM 5 Exciter confocal microscope (Carl Zeiss). The fluorochrome Alexa 488 was excited at 488 nm and the emission fluorescence was recorded between 500 and 550 nm. ImageJ software was used for the analysis of the images. Mean grey values of the nuclei were measured for calculation of staining intensity.

1.8. Treatments with pharmacological inhibitors or inductors

1.8.1. p38 pathways

For inhibition of p38-dependent pathways cells were treated with two well recognized pharmacological inhibitors: SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4- pyridyl)imidazole] (Badger, Bradbeer et al. 1996) and PD169316 [4-[5-(4-fluorophenyl)-2-(4-nitrophenyl)-1H-imidazol-4-yl]-pyridine] (Calbiochem Nottingham, United Kingdom) (Kummer, Rao et al. 1997). Cells were treated for 24 h with each inhibitor at a concentration ranging from 100 nM to 5 µM.

1.8.2. ERK1/2 pathways

In relation to ERK1/2, we used the PD098059 [2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one] (Calbiochem)(Alessi, Cuenda et al. 1995) for 24 hours at concentrations of 1µM, 5 µM or 25 µM.

1.8.3. Caspase-8

For this purpose we used Z-IETD-FMK (Alexis Corporation), a cell permeable non-toxic and irreversible caspase-8 inhibitor. Cells were preincubated for 24 hours in the presence Z-IETD-FMK at concentrations ranging from 5 to 25 µM.

1.8.4. XIAP inhibition

Cells were treated with the XIAP inhibitor BV-6 (Selleckchem) at 3.12 µM, 6.25 µM and 12.5 µM for 48 hours. Thereafter cells were washed once with Hank's solution and assayed for viability (MTT assay).

1.8.5. MAO inhibitors

We tested the effect of chlorgyline and deprenyl (Sigma), two well-characterized inhibitors of MAO-A and MAO-B respectively (Marcocci, De Marchi et al. 2002) on basal DNA fragmentation in wild-type and Nrf2KO MEFs. Cells were treated for 24 hours at 1 μM concentration of chlorgyline and 1 and 10 μM of deprenyl.

1.8.6. Induction of Nrf2 by tBHQ treatment

The Nrf2 nuclear translocation was calculated as the ratio of nuclear Nrf2 levels divided by cytosolic Nrf2 levels obtained by western blotting and immunodetection. To measure nuclear translocation of Nrf2, cells were treated for 6 hours with the Nrf2 inducer tert-butylhidroquinone (tBHQ) (Sigma) prepared at 50 μM in DMSO. The same volume of DMSO (vehicle) was added to the controls. Cytosolic and nuclear fractions were then obtained following the protocol indicated above and the samples were prepared for western blotting and immunostaining with Nrf2 antibody.

1.9. Cell fractionation

1.9.1. Preparation of whole cell extracts from MEFs

Cells were separated from culture dishes using a trypsin-EDTA detaching solution at room temperature. Subsequent extraction procedures were carried out at 4°C. MEFs were disrupted by homogenization in radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl 50 mM, pH 8; NaCl 150 mM; 0.5 % deoxycholate; 0.1 % SDS; 1 mM DTT; 1 % Triton X-100) containing 20 μg/ml of each of the following protease inhibitors: chymostatin, leupeptin, antipain and pepstatin A (CLAP), 1 mM of phenylmethanesulfonylfluoride (PMSF) and phosphatase inhibitor cocktail 2 and 3 (Sigma) at 1:100 dilution. Prior to extraction, cells were centrifuged at 500 x g for 5 minutes, washed with PBS and centrifuged again under the same conditions. The cell pellet was resuspended in RIPA buffer and, after agitation the suspension was centrifuged at 10,000 x g for 15 minutes. Supernatants were saved for determination of protein levels by western blot (see below).

1.9.2. Preparation of cytosolic and nuclear extracts

All extraction procedures were carried out at 4°C. About 2-10 x 10^6 cells were recovered by centrifugation at 500 x g for 5 minutes and washed with cold PBS twice. The cell pellets were resuspended in a hypotonic buffer (10mM HEPES pH 7.9, containing 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 1 mM PMSF, 20 μg/ml CLAP and phosphatase inhibitor cocktail 2 and 3 at 1:100 dilution). Cells were incubated for 15 minutes at 4°C and then, NP40 was added to a final concentration of 0.5%. After a brief vortexing, the cell extracts were

centrifuged at 2,800 x *g* in a tabletop microfuge. Supernatants so obtained constitute crude cytosolic fractions, which were centrifuged again at 135,000 x *g* in a Beckman Optima TLX ultracentrifuge for further purification. The used rotor was the TL4-100.4. The pellets from the 2,800 x *g* centrifugation, containing the nuclei, were resuspeded in a high-salt buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25%v/v Glycerol, 0.5 mM PMSF, 20 µg/ml CLAP and phosphatase inhibitor cocktail 2 and 3 at a 1:100 dilution). Nuclei were incubated at 4°C with a vigorous agitation. Nuclear extracts were recovered after centrifugation for 10 minutes at 10,000 x *g* in a tabletop microfuge at 4°C. All the procedures were carried out at 4°C in order to prevent protein degradation.

1.10. Determination of protein concentration

In order to determine the protein concentration of all the samples, we have performed the method described by Stoscheck (Stoscheck 1990), a variation of the protein assay described by Bradford (Bradford 1976). Triplicates of our samples were taken up to 20 µl. In order to facilitate solubilization, samples were mixed with 50 µl of 1N NaOH. Finally 1 ml of Bradford reagent was added (Bio-Rad). After being incubated for 10 minutes at RT and in the dark, the absorbance of each sample at 595 nm was measured in a DU-640 spectrophotometer (Beckman Coulter).

A standard curve with known concentrations of γ-globulin (from 0 to 20 µg/µl) was used. The absorbance of all samples was kept within the linear range of the standard curve.

1.11. Protein electrophoresis and Western blotting

1.11.1. Sample preparation

Nuclear fractions obtained as described above were used to determine AIF levels, whereas cytochrome *c* was determined in cytosolic fractions. The rest of proteins were determined in whole cells extracts. The required amount of sample was mixed with 1X SDS-dithiothreitol loading buffer (final concentrations: 10% sucrose, 2 mM EDTA, 1.5% (w/v) SDS, 20 mM dithiothreitol, 0.01% (w/v) bromophenol blue and 60 mM Tris-HCl, pH 6.8, freshly prepared from a 5X stock solution that was stored frozen) and boiled for 5 minutes. Samples were then spun shortly before electrophoretic separation of proteins. For the membrane protein Fis1 the required amount of sample was mixed with a mixture composed of 1X loading buffer, CLAP (5 mg/ml) and PMSF (100 mM) in proportions 18:1:1. Heating of the samples was limited to 45°C for 15 minutes to avoid aggregations of membrane proteins. Samples were then centrifuged briefly before gel electrophoresis.

1.11.2. Electrophoresis

TGX 4-20% polyacrylamide gradient gels (Bio-Rad, Hercules, California, USA) were used for all determinations of protein abundance by western blot. One well of each gel was used to load the molecular weight markers (Dual Color Precision Plus Protein Standards, Bio-Rad).

Proteins were separated by electrophoresis in a Criterion system (Bio-Rad) at a constant voltage of 200V (I_{max} 150 mA) for about 45 minutes.

1.11.3. Western blot transfer and loading control

Once the electrophoresis was completed, proteins were transferred to nitrocellulose membranes (Biotrace NT, Pall Corporation, Nueva York, USA) included in the Trans-blot Turbo kit (Bio-Rad). Protein transfer was carried out with a program of 25 V (I_{max} de 2.5A) for 7 minutes.

After completion of the transfer step, membranes were stained with Ponceau S (0.1 % Ponceau S red dye diluted in 1% acetic acid) for 5 minutes at room temperature. The excess of Ponceau S staining was removed by washing with 1% acetic acid. The obtained protein pattern was digitalized in a GS-800 densitometer (Bio-Rad) and used later as protein loading control. Then, the membrane was destained from Ponceau S and blocked with TTBSL buffer (50 mM Tris-HCl pH 7.6, 0.85% NaCl, 0.05% Tween 20 and 5% skimmed milk powder) in two incubation steps with a minimal duration of 30 minutes at room temperature.

1.11.4. Immunostaining, developing and quantification

After the blocking step the membrane was stained with the corresponding primary antibody at 4°C overnight with gentle rotary agitation. Each primary antibody was diluted in TTBSL at the concentration indicated in table 1. After incubation with the corresponding primary antibody the membrane was washed three times at room temperature with gentle rotary agitation for 5 minutes with TTBS (50 mM Tris-HCl pH 7.6, 0.85% NaCl, 0.05% Tween 20). Afterwards, the corresponding species-specific secondary antibody diluted in TTBSL (1:2,000) and coupled to horseradish peroxidase was added and the membrane incubated at room temperature and in agitation for 1 hour. Then, the antibody was removed and the membrane washed three times with TTBS at room temperature for 5 minutes. Finally, the membrane was washed once for 10 minutes with TBS buffer (50 mM Tris-HCl pH 7.6, 0.85% NaCl).

Developing of images was performed by enhanced chemiluminescence (ECL Plus, Amersham Bioscience, Uppsala, Sweden). Luminescence derived from the horseradish peroxidase-catalyzed reaction was recorded with Kodak Biomax XAR films (Carestream Health, Rochester, New York, USA). Photographic films obtained after ECL autoradiography were scanned in a BioRad GS800 densitometer to obtain digital images for quantification of intensity reaction using Quantity One software (BioRad). Data obtained from quantification of stained bands (in arbitrary units) were normalized to that of the corresponding lane stained with Ponceau S to correct for minor differences in protein loading between samples. The corresponding ponceau S to the western blots appear in the Appendix A: Loading Control of Western Blots.

Primary antibody	Dilution	Reference	Secondary antibody	Dilution	Reference
AIF	1:500	SC-9416	Goat anti-IgG	1:2000	Sigma A-5420
Akt	1:1000	SC-8312	Rabbit anti-IgG	1:2000	SC-2004
Apaf-1	1:1000	SC-33879	Goat anti-IgG	1:2000	Sigma A-5420
Bax	1:1000	SC-7480	Mouse anti-IgG	1:2000	Sigma A-9044
Bcl-2	1:250	SC-492	Rabbit anti-IgG	1:2000	SC-2004
Bid	1:500	SC-11423	Rabbit anti-IgG	1:2000	SC-2004
Caspase-3	1:1000	9662	Rabbit anti-IgG	1:2000	SC-2004
Caspase-8	1:1000	4790	Rabbit anti-IgG	1:2000	SC-2004
Caspase-9	1:1000	9504	Rabbit anti-IgG	1:5000	SC-2004
Cytochrome *c*	1:250	Ph 65981A	Mouse anti-IgG	1:2000	Sigma A-9044
Drp1	1:1000	SC-32898	Rabbit anti-IgG	1:2000	SC-2004
ERK1/2	1:1000	442704	Rabbit anti-IgG	1:2000	SC-2004
Fis1	1:1000	SC-98900	Rabbit anti-IgG	1:2000	SC-2004
Glut1	1:1000	SC-7903	Rabbit anti-IgG	1:2000	SC-2004
JNK1	1:1000	SC-1648	Mouse anti-IgG	1:2000	Sigma A-9044
MAO-B	1:1000	Sigma 1821	Rabbit anti-IgG	1:2000	SC-2004
Mfn1	1:1000	SC-50330	Rabbit anti-IgG	1:2000	SC-2004
Mfn2	1:1000	SC-50331	Rabbit anti-IgG	1:2000	SC-2004
Nrf2	1:1000	SC-722	Rabbit anti-IgG	1:2000	SC-2004
Opa1	1:1000	SC-30573	Goat anti-IgG	1:2000	Sigma A-5420
p38	1:2000	SC-535	Rabbit anti-IgG	1:2000	SC-2004
P62 (SQSTM1)	1:500	SC-25575	Rabbit anti-IgG	1:2000	SC-2004

PGC1α	1:1000	SC-13097	Rabbit anti-IgG	1:2000	SC-2004
Phosho-Akt	1:1000	SC-7985	Rabbit anti-IgG	1:2000	SC-2004
Phospho-Bcl-2	1:1000	SC-16323	Goat anti-IgG	1:2000	Sigma A-5420
Phospho-ERK1/2	1:1000	SC-7383	Mouse anti-IgG	1:2000	Sigma A-9044
Phospho-JNK1	1:1000	SC-6254	Mouse anti-IgG	1:2000	Sigma A-9044
Phospho-p38	1:1000	SC-7975	Rabbit anti-IgG	1:2000	SC-2004
Trx1	1:1000		Goat anti-IgG	1:2000	Sigma A-5420
XIAP	1:1000	SC-8780	Goat anti-IgG	1:2000	Sigma A-5420

Table 1: Antibodies used for immunodetection in western blotting. Each primary antibody was used in combination with the corresponding secondary antibody as shown in the same lane. References denoting "SC" are from Santa Cruz Biotechnologies (Santa Cruz, California, USA). Anti-cytochrome *c* antibody is from Pharmingen that now belongs to BD Biosciences (San José, California, USA). MAO-B antibody and mouse and goat secondary antibody are from Sigma-Aldrich. Anti-caspases antibodies are from Cell Signaling Technology (Spain). ERK1/2 antibody is from EMD Millipore (Spain). Trx1 antibody was kindly provided by José Antonio Bárcena (University of Córdoba).

1.11.5. Phosphorylated/total protein ratio

For the MAP kinases, Akt and Bcl-2 proteins, after obtaining the levels of phosphorylated proteins by western blot and immunodetection as previously described, membranes were subjected to stripping of bound antibodies by washing the membrane twice for 15 minutes with a stripping buffer (62 mM Tris pH 6.7, 2% w/v SDS, 1.4% V/V β-Mercapto-ethanol) at 70°C. Thereafter, membranes were washed twice with distillated water. Then, membranes were incubated with the primary and the corresponding secondary antibody as previously described for determining total levels of the protein (phosphorylated plus nonphosphorylated) by immunodetection. Data obtained from quantification of stained phosphorylated protein bands (in arbitrary units) were normalized to the total levels of protein to establish putative alterations in its phosphorylation degree.

1.12. Quantification of DNA fragmentation (Apoptotic index)

A photometric enzyme-immunoassay was performed in immortalized MEFs for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA-fragments (mono- and oligonucleosomes) released from the nucleus to the cytosol of cells undergoing apoptosis. DNA fragmentation extent was quantified by using the ELISA kit developed by Roche Diagnostics (Mannheim, Germany). Manufacturer´s recommendations were followed. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the

streptavidin-coated plate *via* its biotinylation. Additionally, the anti-DNA antibody conjugated with peroxidase activity reacts with the DNA-component of the nucleosomes. After the two hour incubation, three washing steps were carried out in order to eliminate antibody not bound to streptavidine. Plates were incubated with the developing reactive and the reaction was stopped after 20 minutes.

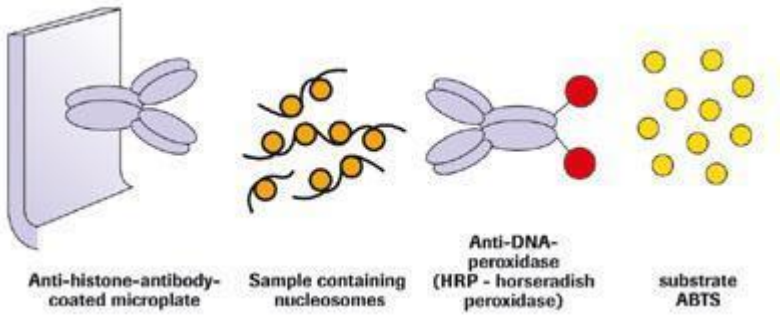

Figure 11: Summary of Cell Death Detection ELISA kit reaction principles. Two different antibodies detect the nucleosome components and bind to the plate and to the reaction buffer. The image has been taken from Roche Diagnosis.

Absorbance was determined at 405 nm using a Flex Station 3 (Molecular Devices, Sunnyvale, CA, USA) and data were reported as arbitrary OD units/mg cytosolic protein.

Assays of DNA fragmentation were carried out as described above on untreated cells to estimate basal levels of apoptosis in cell cultures. In some cases DNA fragmentation was also measured in cells treated with different inhibitors to test the putative participation of different pathways in apoptosis induction.

1.13. Caspase activity assay

Activities of caspases-8, -9 and -3 were determined in cytosolic fractions obtained from immortalized MEFs. Proteolytic activity of each caspase was determined by fluorimetry in an assay medium containing 25 mM HEPES-KOH buffer (pH 7.4), 10% sucrose, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 5 mM DTT and 200 µg of cytosolic protein in a final volume of 200 µl. Samples were preincubated for 25 minutes at 37°C in the assay medium and specific fluorogenic substrates were then added to a final concentration of 40 µM. Samples were incubated for 1 hour at 37°C in the dark. The following substrates (obtained from Alexis Corporation, San Diego, CA, USA) were used: Ac-DMQD-AMC (caspase-3), Ac-IETD-AMC (capase-8), and Ac-LEHD-AMC (caspase-9). After incubation, the reaction was stopped by adding 20 µl of 1N HCl. The mixture was diluted with 1.5 ml water and fluorescence signal was then recorded with an Aminco-Bowman Series 2 Luminiscence Spectrometer set at wavelengths of 380 nm (excitation) and 460 nm (emission). Assays were carried out both

in the absence and in the presence of the corresponding specific inhibitor for each caspase. The following inhibitors (Alexis Corporation, San Diego, CA, USA) were used at a final concentration of 83 μM: Ac-DMQD-CHO (caspase-3); Ac-IETD-CHO (capase-8); Ac-LEHD-CHO (caspase-9). Caspase activities were calculated from the difference of fluorescence measurements obtained in the presence and in the absence of the corresponding inhibitor. Specific activities were expressed as arbitrary units normalized to the mg of protein

2. Animal model

Five months old male wild-type (Wt) mice, NQO1KO mice and Nrf2KO mice on C57BL/6 background were housed at the Gerontology Research Center, in Baltimore, MD, two to four animals per cage with *ad libitum* access to mouse chow (Harlan Teklad, Madison, WI, USA) and tap water. Animal rooms were maintained at 20-22 ºC with a 12-h light/dark cycle. All animal protocols were approved by the Animal Care and Use Committee (352-TGB-2015) of the National Institute on Aging (National Institutes of Health). Animals were sacrificed by cervical dislocation at National Institute on Aging (NIH).

2.1. Organ harvesting and preparation of total homogenates from mouse tissues

After euthanization of mice, the organs were excised and quickly frozen in liquid nitrogen. 50 mg of tissues were weighted and the tissue samples were lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (as indicated for MEFs extracts) using a mechanical, motor-driven homogenizer (Ultra-Turrax T25, IKA, Staufen, Alemania). Lysates were centrifuged at maximum speed for 15 minutes in a tabletop microfuge. Supernatants were transferred to new vials and conserved at 80°C. All the procedures were carried out at 4°C in order to prevent protein degradation.

2.2. Preparation of cytosolic and nuclear extracts from liver

To isolate the cytosolic and the nuclear fractions from liver the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, USA) was used in accordance with the manufacturer's instructions. All the procedures were carried out at 4°C in order to prevent protein degradation.

2.3. Nrf2 induction by tBHQ induction

Male mice of Wt and NQO1KO genotypes were injected intraperitoneally with tBHQ at a concentration of 50 mg/kg in PBS containing 1% DMSO. Controls were also injected with same amount of the vehicle. After 6 hours mice were sacrificed by cervical dislocation.

Organs were collected and snap frozen in liquid nitrogen. Cytosolic and nuclear fractions from livers were obtained as already described.

2.4. MEFs inoculation in nude mice

About 1.5 million of MEFs were injected per nude mouse. B10F16 cells from mouse skin melanoma were used as a positive control for tumor generation. 26 days after the injections animals were sacrificed and the tumors excised. This procedure was carried out in the Gerontology Research Center, National Institute of Health (Baltimore, MD).

2.5. Histological analysis of teratomas

To study the histopathology of teratomas, the tumors were fixed and embedded in paraffin following routine histological procedures. This process was carried out at the National Institutes on Aging (NIH, Baltimore).

The slides containing paraffin sections were deparaffinized in xylene for 15 minutes. The sections were then rehydrated using decreasing concentrations of ethanol. Rehydration was accomplished by immersing the sections sequentially in 100% ethanol for 5 minutes, 90% ethanol for 5 minutes, and finally in 70% ethanol for 5 minutes. Slides were then washed with water for 5 minutes. We used haematoxylin-eosin for staining of the sections. Slides were placed into haematoxylin dye solution for 20 minutes, rinsed with water for approximately 15 minutes, and subsequently submerged in eosin for 5 minutes. After a final rinsing step with water to remove any excess of unbound dye, dehydration of the sections was carried out with the sequence: 70% ethanol, 90% ethanol and 100% ethanol. Sections were then cleared with xylene and mounted with coverslips using Permount (xylene based) (Sigma). Digital images were obtained in a Leica DM5000 B microscope.

3. Statistical analysis

Data were analyzed and graphs generated using Graphpad Prism 5 (Graphpad Software Inc, San Diego, CA, USA). Data shown in this work are means ± standard error of the mean (SEM) from at least three different determinations. Comparisons between genotypes were assessed using two-tailed Student´s t test. In case variances were not equal Welch correction was applied. In case a parametric test was not applicable, the Mann-Whitney test was used. Different treatments within a genotype were compared with the one-way ANOVA analysis and the Dunnet posthoc test. In order to compare the combined effect of genotype and treatment, two-way ANOVA analysis was used. Differences were considered significant with * $p< 0.05$, ** $p<0.01$ or *** $p<0.001$. Between 0.05 and 0.1, p values were considered a trend.

Results

1. MEFs lacking nuclear factor (erythroid-derived 2)-like 2 (Nrf2)

Our group have previously shown that cultures of Nrf2KO MEFs displayed a stabilized doubling rate between passages 20 and 52 but then, the cells underwent a sharp decline in growth rate and could not divide beyond passage 54. In contrast, wild-type MEFs were able to grow steadily until passage 62, although they could not surpass passage 64 (Jodar, Mercken et al. 2011). For the determinations described in this chapter we used MEFs obtained from passages 25 to 35.

1.1. Thymidine incorporation assay

When comparing doubling rates of cultures from these two genotypes, we previously showed that Nrf2KO MEFs exhibited a slight, although statistically significant, decrease in growth rate in comparison with the wild-type controls (Jodar, Mercken et al. 2011). Thus, we aimed to confirm these results using a different proliferation assay. For this purpose we used radiolabeled thymidine in order to measure the incorporation of [^{3}H]-thymidine into proliferating cells.

Our results were fully consistent with these previous observations, since we found out that the level of incorporated [^{3}H]-thymidine was significantly lower for Nrf2-null immortalized MEFs in comparison with their wild-type counterparts (Figure 12). This indicates that the DNA synthesis and, in turn, the proliferation rate, is lower for these cells.

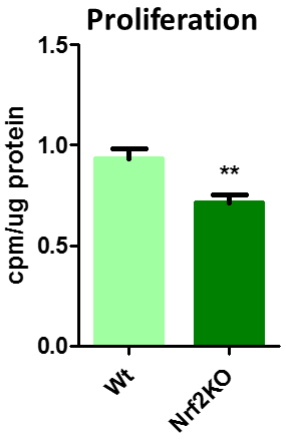

Figure 12: Proliferation assay in wild type and Nrf2KO MEFs. Cells were cultured for 24 hours in the presence of 0.25 µCi/ml [3H]- thymidine. After washing, macromolecules (including DNA) were precipitated with TCA and the radioactivity measured in the TCA-insoluble fraction. Levels of incorporated [^{3}H] thymidine were significantly lower for Nrf2-null immortalized MEFs in comparison with their Wt counterparts (**p<0.01). Data are means ± SEM (n=6).

1.2. Basal apoptosis levels

Cell growth is the balance between proliferation and cell death. Thus, we quantified basal apoptosis levels in Wt and Nrf2KO MEFs by ELISA determination of oligonucleosomal chromatin fragments released to the cytosol. This method was chosen because of its high sensitivity, and also because it allows to easily distinguish apoptosis from necrotic cell death, since during the latter oligonucleosomal fragments are not retained in cytosol but released to the culture medium. As depicted in Figure 13A, the abundance of oligonucleosomal fragments retained in the cytosolic compartment was greatly increased in Nrf2KO MEFs, indicating a significant activation of basal apoptosis in cells lacking Nrf2. Since the levels of basal apoptosis show a high dependence on cell density in MEFs (Porras, Zuluaga et al. 2004), we also measured cytosolic oligonucleosomal fragments in MEFs cultured at varying cellular densities. As depicted in Figure 13B, DNA fragmentation was always higher in Nrf2KO cells independently of culture density. Nrf2 signaling and pathophysiology in liver have been widely linked, Nrf2 playing a role in carcinogenesis and other chronic diseases (reviewed by (Shin, Yang et al. 2013)). In addition, oncogenesis has been also shown particularly sensitive to alterations in Nrf2 status in lung (Suzuki, Shibata et al. 2013). For these reasons we aimed to detect levels of oligonucleosomal fragments in liver and lung samples obtained from wild-type and Nrf2KO mice and demonstrated that apoptotic DNA fragmentation was also increased in Nrf2KO mice (Figure 13C and Figure 13D).

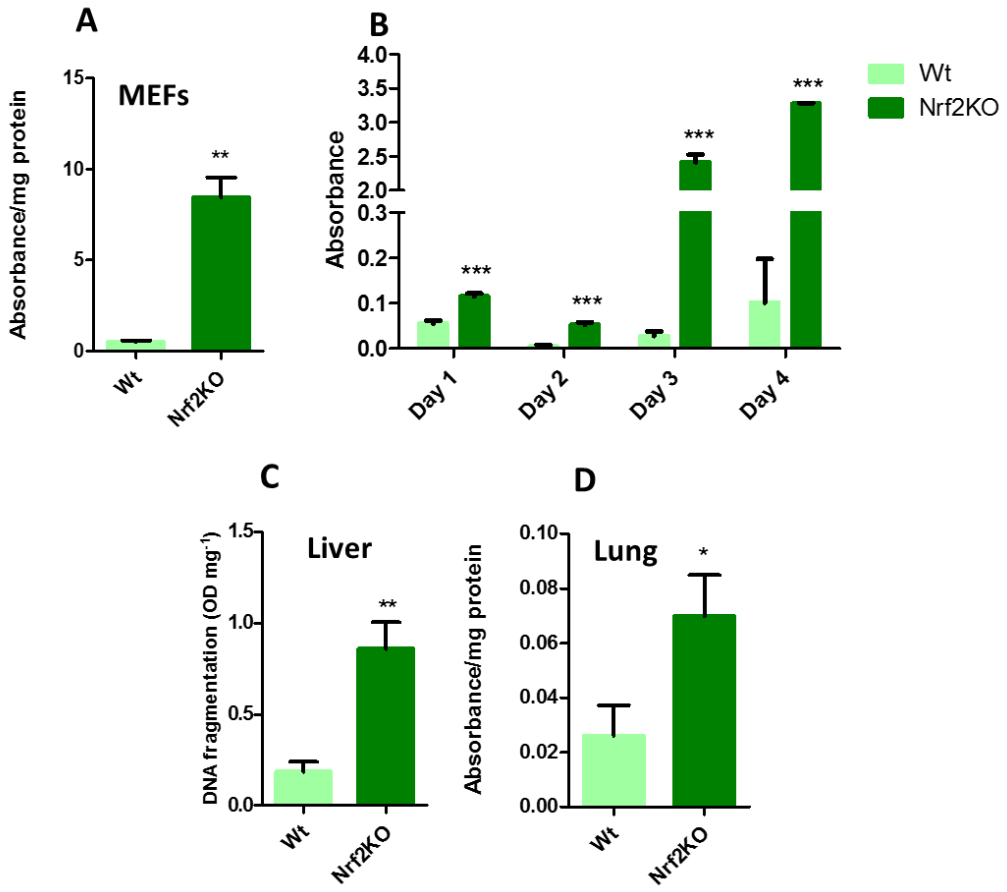

Figure 13: Apoptotic Index. A) MEFs: We found out that apoptosis was increased in the Nrf2KO immortalized MEFs when compared to their Wt counterparts (**p<0.01). Data are means ± SEM (n=6). **B) Effect of culture density in MEFs:** Apoptotic index was higher in the Nrf2KO when compared to their wild-type counterparts irrespective of culture density (***p<0.001) (two-ways ANOVA). Data are means ± SEM (n=3). **C) Liver:** Levels of oligonucleosomal fragments were higher in Nrf2KO liver (**p<0.01). Data are means ± SEM (n=5). **D) Lung:** DNA fragmentation was also significantly increased in lung tissue from Nrf2KO mice (*p<0.05). Data are means ± SEM (n=5).

1.3. Phosphorylated histone H2AX

H2A.X is a histone variant which is ubiquitously distributed throughout the genome. The use of antibodies capable of recognizing histone H2A.X that is phosphorylated at serine 139 (γH2A.X) in immunocytochemistry is a specific indicator for the existence of a DNA double strand breaks (Takahashi, Mori et al. 2008). Thus, we aimed to assess the levels of histone H2A.X phosphorylation as an additional independent way of measuring DNA damage in wild-type and Nrf2KO MEFs. As depicted in Figure 14A, we found an increase in the intensity of the H2A.X staining in the Nrf2KO MEFs when compared to their wild-type counterparts. Quantification of mean grey values with ImageJ software confirmed the existence of increased DNA damage in MEFs lacking Nrf2 (Figure 14B).

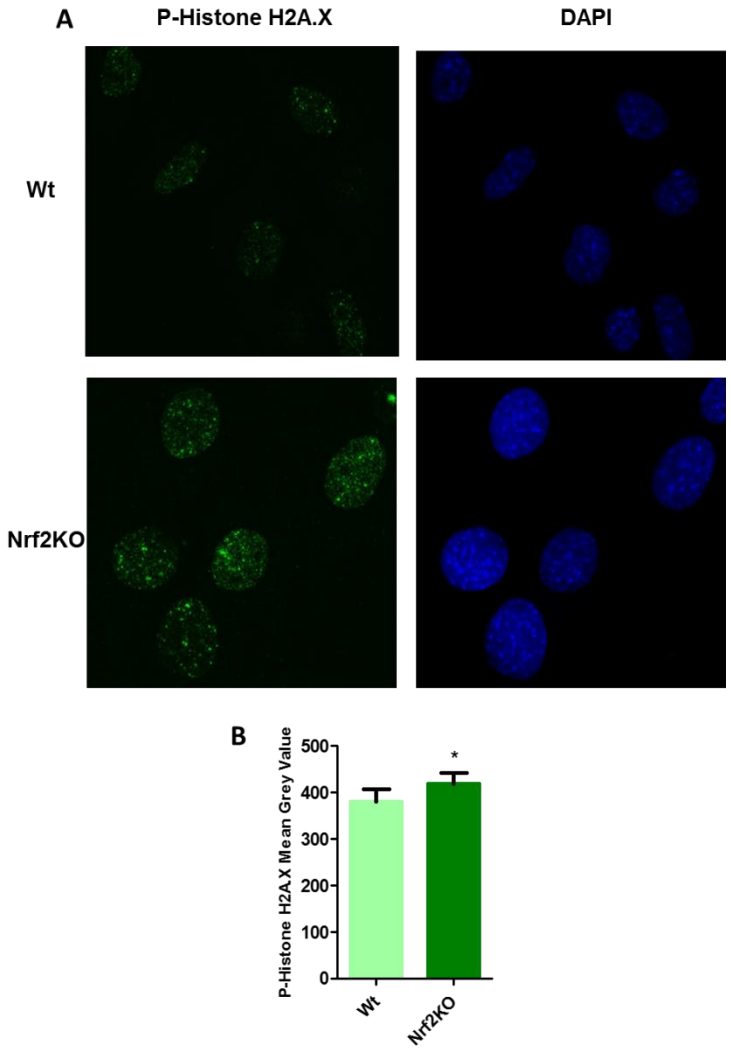

Figure 14: Phosphorylated H2A.X (Ser139). A) Representative images of DAPI and P-H2A.X. Nuclei of Nrf2KO MEFs are more strongly stained with an antibody raised against P-H2A.X. **B) Quantification of fluorescence intensity:** Nrf2KO MEFs showed a higher mean grey value of γH2A.X staining than wild-type MEFs (**p*<0.05). Data are means ± SEM (n=60).

1.4. Mitochondrial apoptotic signaling

As already indicated in the Introduction section, one of the most important events in the apoptotic process is the permeabilization of the OMM that allows the release of several pro-apoptotic factors like cytochrome *c* and AIF to trigger the apoptotic cascade.

1.4.1. Bcl-2 and Bax polypeptides

To gain insights about the cellular mechanisms that could be involved in the activation of basal apoptosis in cells lacking Nrf2 we first evaluated the levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic polypeptide Bax in wild-type and Nrf2KO MEFs by western blot and immunodetection. As depicted in Figure 15A and Figure 15B, the abundance of Bcl-2 was greatly diminished in the KOs compared with wild-type MEFs, with

no significant change being observed for Bax. Since the Bcl-2/Bax ratio has been proposed as an indicator of cell fate towards survival or apoptosis (Oltvai, Milliman et al. 1993), we calculated Bcl-2/Bax ratio values from the optical densities obtained from the corresponding western blots stained with each antibody. We observed that a substantial decrease of Bcl-2/Bax ratio was maintained in Nrf2KO cells (Figure 15C), indicating the basal activation of pro-apoptotic events in cells lacking Nrf2.

1.4.2. Proapoptotic mitochondrial factors: cytochrome *c* and AIF

The balance between pro- and anti-apoptotic members of the Bcl-2 family defines the permeabilization of the OMM and the release of pro-apoptotic mitochondrial factors that trigger the apoptotic signaling cascade. Thus, we next determined by western blot and immunodetection in wild-type and Nrf2KO MEFs the levels of cytosolic cytochrome *c* as an estimate of its release/accumulation form the mitochondrial IMS into the cytosol, and the levels of nuclear AIF as an estimate of its release from the mitochondria and its further accumulation into the nucleus. In accordance with the decreased Bcl-2 levels and Bcl-2/Bax ratio, cytosolic cytochrome *c* and nuclear AIF were dramatically increased in Nrf2KO MFEs (Figure 15D and Figure 15E), indicating enhanced permeabilization of the outer mitochondrial membrane in cells lacking Nrf2.

1.4.3. Apaf-1

We also evaluated how genetic deletion of Nrf2 affected Apaf-1 levels. As indicated above, cytochrome *c* release is a crucial event of the intrinsic pathway by activating another adaptor protein, known as apoptotic protease-activating factor-1 (Apaf-1) which, together with dATP and cytochrome *c*, the apoptosome to recruit and activate caspase-9, and subsequently caspase-3 (Bratton and Salvesen 2010). Interestingly, we found that this pro-apoptotic adaptor protein was also increased in Nrf2KO MEFs (Figure 15F).

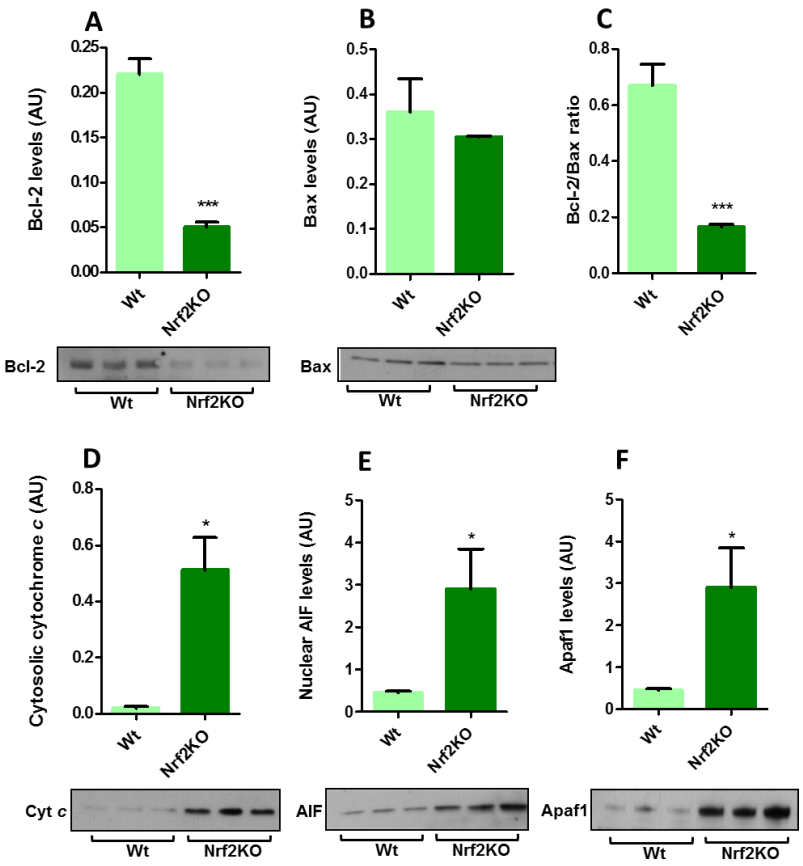

Figure 15: Markers of mitochondrial apoptotic pathway. A) Bcl-2 levels: We found out that the levels of Bcl-2 were decreased in the Nrf2KO immortalized MEFs if comparison with their Wt counterparts (***p<0.001). **B) Bax levels:** Levels of Bax polypeptide did not change among Nrf2KO and Wt MEFs. **C) Bcl-2/Bax ratio:** When we calculated the Bcl-2/Bax ratio, we observed a decrease in Nrf2-null immortalized cells (***p<0.001). **D) Cytosolic cytochrome *c*:** The release/accumulation of cytochrome *c* to the cytosol was higher in the Nrf2KO MEFs when compared to their Wt counterparts (*p<0.05). **E) Nuclear AIF:** Levels of nuclear AIF were significantly elevated in Nrf2-null cells (*p<0.05). **F) Apaf-1 levels:** Nrf2KO MEFs exhibited higher levels of Apaf-1 (*p<0.05). Data are means ± SEM (n=3).

1.5. Mitochondrial potential (ΔΨ) alterations and ROS production in Nrf2KO MEFs

Mitochondrial membrane potential was measured from the fluorescence changes of JC-1 probe, as described in Material and Methods section. We found no differences in mitochondrial potential among Nrf2KO and wild-type MEFs (Figure 16A). Also we aimed to assess by flow cytometry the levels of intracellular peroxides and we have observed no differences between both genotypes (Figure 16B).

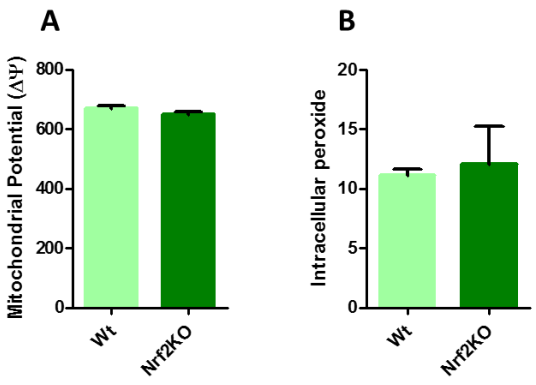

Figure 16: Mitochondrial potential and intracellular peroxide levels. A) Mitochondrial potential: Mitochondrial potential changes did not reach significant differences among Nrf2KO and wild-type MEFs. **B) Intracellular peroxide levels:** The levels of the intracellular peroxide did not change in Nrf2KO MEFs in relation to their wild-types MEFs.

1.6. Pro-caspases and activated caspases, truncated Bid and XIAP

With the purpose of elucidate the apoptotic signaling pathways involved in activation of basal apoptosis in Nrf2KO MEFs; we next analyzed how deletion of Nrf2 affected activities of different caspases. This analysis was first carried out by measuring their enzymatic activities by a fluorometry assay using fluorogenic peptides as substrates. We measured enzyme activities of caspase-8, which is activated *via* the extrinsic pathway, caspase-9, a key factor of mitochondrial or intrinsic pathway apoptosis, and caspase-3 activity, the most representative executioner caspase in which both extrinsic and intrinsic pathway converge. As depicted in Figure 17A and Figure 17B, proteolytic activities of caspases-9 and -3 were not increased significantly in Nrf2KO MEFs in comparison with the control cells, despite the substantial decrease of Bcl-2/Bax ratio and the elevation of cytosolic cytochrome *c* and Apaf-1 levels in the KOs. On the other hand, when we analyzed the enzymatic activity of caspase-8, a significant increase was observed for Nrf2KO cells compared with their wild-type counterparts (Figure 17C).

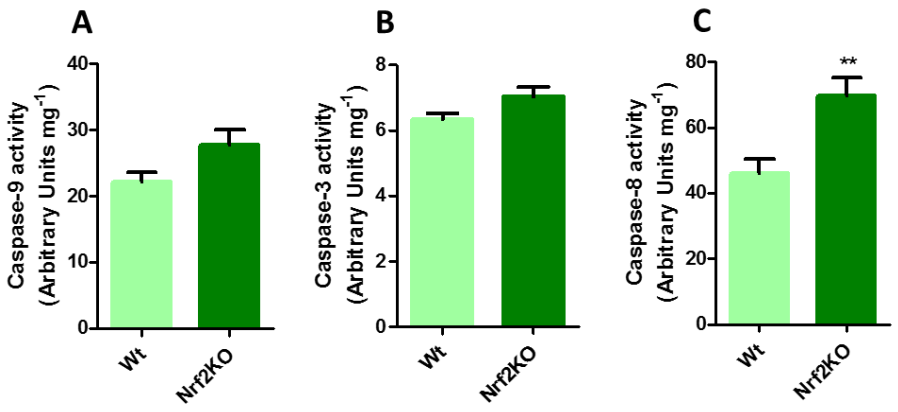

Figure 17: Enzymatic activities of caspases. A) Caspase-9 activity: Proteolytic activity of caspases-9 was not increased significantly in Nrf2KO MEFs in comparison with the control cells. **B) Caspase-3 activity:** Enzymatic activity of caspase-3 did not change in Nrf2KO cells in comparison to Wt counterparts. **C) Caspase-8 activity:** when we analyzed the enzymatic activity of caspase-8, a significant increase was observed for Nrf2KO cells compared with their wild-type counterparts (**p< 0.01). Data are means ± SEM (n=5).

To confirm the results obtained using the enzymatic assay, caspase activation was also studied by western blot determination of pro-caspases and their corresponding cleaved activated caspases. In the case of procaspases levels, we found that the level of procaspase-9 was substantially increased in Nrf2KO MEFs (Figure 18A), procaspase-3 was maintained (Figure 18B), and procaspase-8 was slightly decreased (Figure 18C).

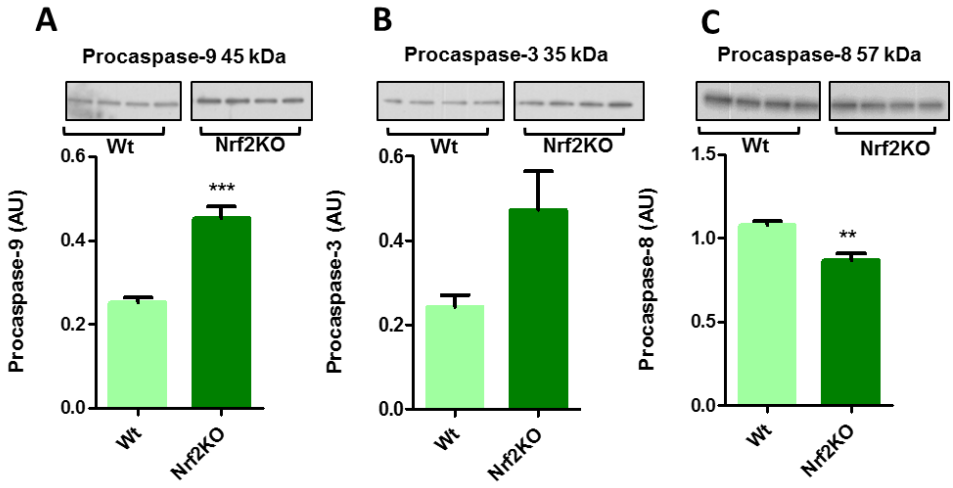

Figure 18: Levels of procaspases. A) Procaspase-9: The level of procaspase-9 was substantially increased in Nrf2KO MEFs (***p< 0.001). **B) Procaspase-3:** Levels of procaspases-3 were maintained in Nrf2KO MEFs in comparison with the wild-type controls. **C) Procaspase-8:** Levels of procaspase-8 were slightly decreased in Nrf2-null MEFs (**p< 0.01).

In relation with the activated cleaved caspases, we observed a trend towards an increase in the 37/39 kDa activated fragments of caspase-9 in Nrf2KO MEFs (p=0.0726) (Figure 19A). However, no activation of caspase 3 (neither 19 kDa nor 17kDa fragments) was found (Figure 19B and Figure 19C). In agreement with the results obtained by following the enzymatic assay against fluorogenic peptide substrates, levels of cleaved caspase-8 (both 24/26 kDa and 18 kDa activated fragments) were significantly elevated in the KOs (Figure 19D and Figure 19E).

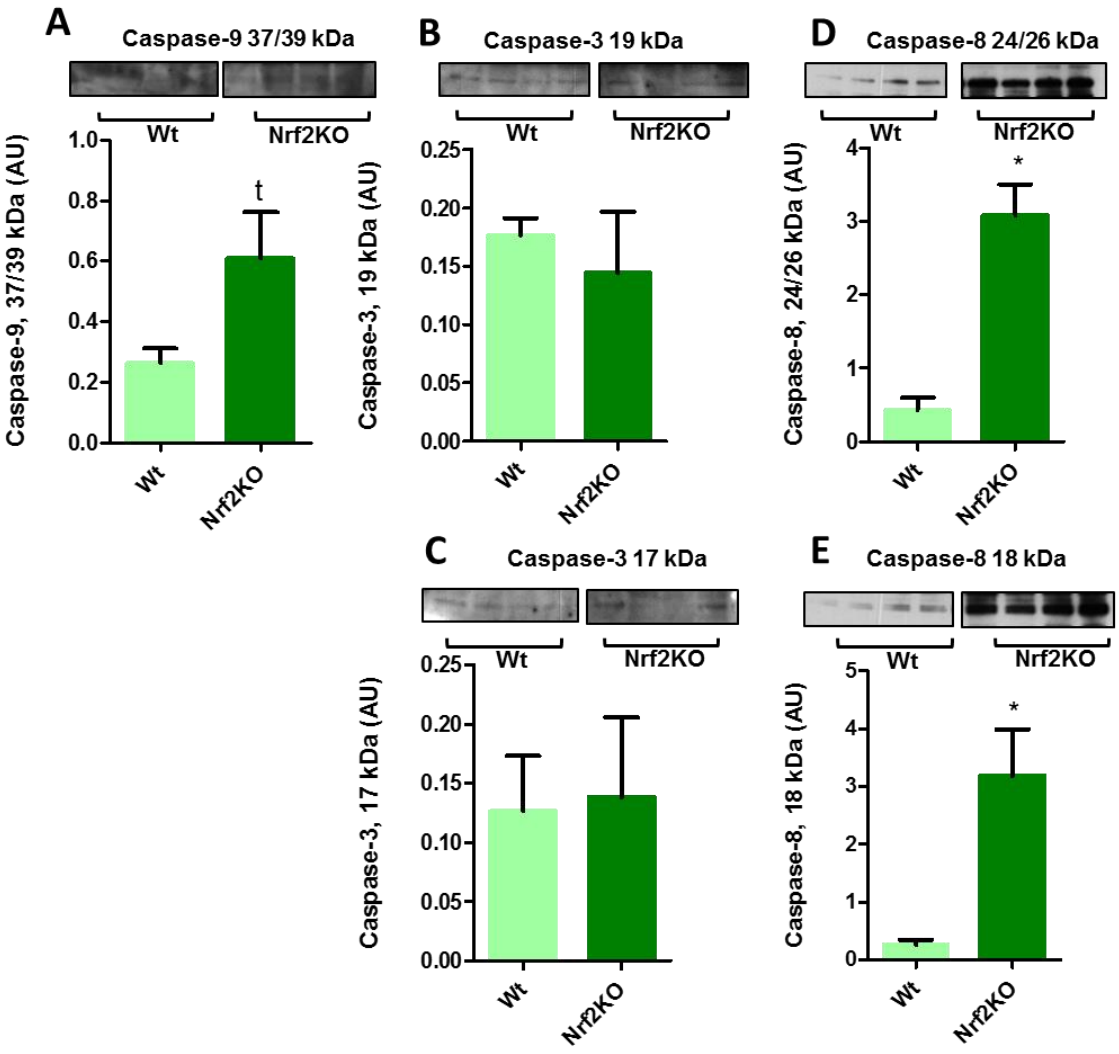

Figure 19: Levels of cleaved caspases. **B) Active caspase-9:** We have observed a slight trend towards an increase of the 37/39 kDa activated fragments of caspase-9 in Nrf2KO MEFs (p=0.0726 with a two-tailed t test). **B) and C) Active caspase-3:** No activation of caspase 3 (neither 19 kDa nor 17kDa fragments) was found in Nrf2KO MEFs. **F) Procaspase-8:** Levels of procaspase-8 were slightly decreased in Nrf2-null MEFs (**p< 0.01). **D) and E) Active caspase-8:** Cleaved, activated caspase-8 levels were significantly elevated, both for the 24/26 kDa and the 18 kDa caspase-8 fragments (*p< 0.05). Data are means ± SEM (n=4).

Since MEFs are well characterized type II cells which transduce apoptotic signaling from the plasma membrane to the mitochondria (Walter, Schmich et al. 2008), we tested if activation of caspase-8 (plasma membrane-dependent) played a significant role in the increase of basal apoptosis in Nrf2KO MEFs. For this purpose we followed two different approaches: (i). to measure Bid and tBid levels in wild-type and Nrf2KO MEFs, and (ii). to test the effect of Z-IETD-FMK, a cell permeable non-toxic and irreversible caspase-8 inhibitor, on apoptotic DNA fragmentation. As depicted in Figure 20A, levels of full-length Bid did not change among Wt and Nrf2KO MEFs. Regarding the abundance of tBid, the cleaved form of Bid was even decreased in the KOs despite their increased levels of cleaved activated caspase-8. For caspase-8 inhibition experiments, cells were preincubated for 24 hours in the presence Z-IETD-FMK at concentrations ranging from 5 to 25 μM. After preincubation with the caspase-8 inhibitor, cells were lysed to obtain cytosolic fractions for the quantification of oligonucleosomal fragments levels by ELISA. As shown in Figure 20B, we observed no effect on the levels of cytosolic DNA oligonucleosomal and basal apoptosis levels remained equally increased in the KOs, both in the absence and in the presence of the caspase-8 inhibitor at any of the tested concentrations.

One of the main IAPs is the X-chromosome linked Inhibitor of Apoptotic Proteases (XIAP), known to behave as a potent inhibitor of caspase-9 and effector caspases (Jost, Grabow et al. 2009). Thus, we also measured XIAP levels in cells from both genotypes. However, XIAP protein levels were found unchanged among wild-type and Nrf2KO MEFs (Figure 20C).

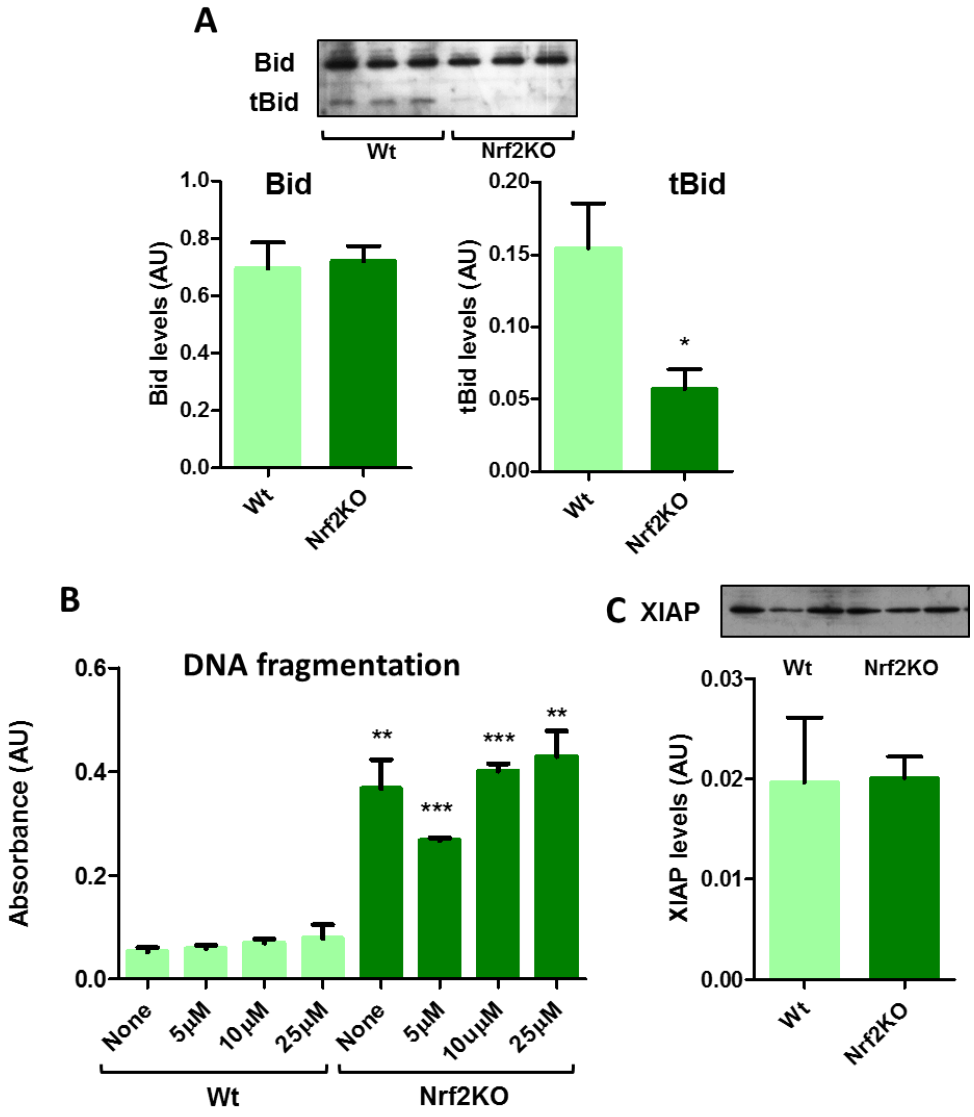

Figure 20: Role of caspase-8 activation in MEFs basal apoptosis and XIAP levels. A) Bid and tBid: Levels of full-length Bid were unchanged among wild-type and Nrf2KO MEFs whereas the abundance of tBid was decreased in the Nrf2KO MEFs (*p< 0.05). Data are means ± SEM (n=3). **B) Caspase-8 inhibitor treatment:** No effect on DNA fragmentation was achieved by treating wild-type or Nrf2KO MEFs with the caspase-8 inhibitor Z-IETD-FMK, and basal apoptosis levels remained equally increased in the KOs both in the absence and in the presence of the inhibitor at any of the tested concentrations (**p<0.01 and ***p<0.0001)**.** Data are means ± SEM (n=4). **C) XIAP levels:** Levels of XIAP did not change between Nrf2KO and wild-type MEFs. Data are means ± SEM (n=3).

1.7. Alterations of MAPK and Akt pathways in Nrf2KO MEFs

We also aimed to investigate if signal transduction pathways involving different Mitogen-activated protein kinases (MAPKs) played a role in the regulation of apoptotic signaling in Nrf2KO MEFs. All eukaryotic cells have several MAPK pathways, each one being activated preferentially by different stimuli. These signaling pathways regulate a variety of cellular processes including proliferation, differentiation, survival, and death (Kim and Choi 2010).

We thus measured phosphorylated and total levels of the main MAPKs, including two stress-activated protein kinases: p38 and JNK1, and the extracellular signal-regulated kinase: ERK1/2 (Figure 21A). Regarding p38, we found that the deletion of Nrf2 resulted in an increase in the activated form of this stress-activated kinase (Figure 21B) although no significant differences were observed in the activation of JNK1 among Nrf2KO and wild-type MEFs (Figure 21C). In relation with ERK1/2, our results showed a significant increase of the ERK1/2 phosphorylated forms in Nrf2KO MEFs (Figure 21D).

Also we wanted to quantify the levels of activated Akt, a protein involved in a pathway related to proliferation and survival, protecting cells against apoptosis (Altomare and Khaled 2012). We found a trend toward decreased levels of phosphorylated Akt in Nrf2KO MEFs although differences did not reach statistical significance (Figure 21E).

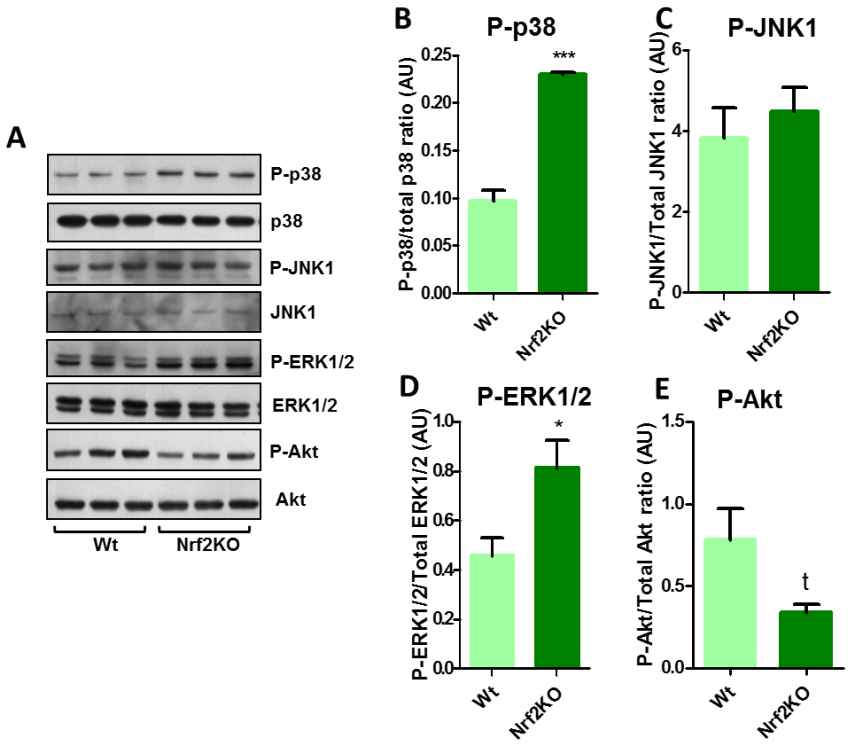

Figure 21: Alterations of MAPK and Akt pathways in Nrf2KO MEFs. A) Western blots showing phosphorylated and total levels of MAPK and Akt: B-E) Calculations of kinase activation by phosphorylation. We measured phosphorylated and total levels p38, JNK1, ERK1/2 and Akt. Putative alterations in the activity of each pathway were estimated from the ratio between the signals corresponding to phosphorylated and total polypeptide levels for each kinases. **B) p38 activation:** We found an increase in the activated form of p38 in Nrf2KO immortalized MEFs (***p<0.001). **C) JNK1 activation:** We found no significant differences between the two genotypes. **D) ERK1/2 activation:** The results showed an increase of the phosphorylated proteins in Nrf2KO (*p<0.05). Data are means ± SEM (n=3). **E) Akt activation:** Changes of

phospho-Akt did not reach significant differences with the two-tailed t-test (p=0.084), although they tended to decrease in Nrf2KO and were significant with a one-tailed t-test. Data are means ± SEM (n=3).

Since phosphorylation of ERK1/2 and p38 MAPKs was significantly increased in Nrf2KO MEFs in comparison with the wild-type controls, we next studied if the activation of these kinases was involved in the increased basal apoptotic levels of Nrf2KO MEFs. For this purpose we tested the effect on DNA fragmentation of well-established pharmacological inhibitors of these two MAPK pathways. The MEK inhibitor PD98059 was used to test the putative participation of ERK1/2, whereas both SB203580 and PD169316 were used to test the involvement of p38 MAPK.

No effect on DNA fragmentation was observed when MEFs (either wild-type or Nrf2KO) were cultured in the presence of PD98059 at a concentration range from 1 to 25 μM (Figure 22A), indicating that activation of ERK1/2 is not related with the regulation of basal apoptosis in the MEFs.

When we studied the effects of the two p38 inhibitors, SB203580 and PD169316, we found that, at some of the concentrations, PD169316 decreased basal DNA fragmentation in wild-type MEFs (Figure 22C), although no effect was observed for SB203580 (Figure 22B). In the case of Nrf2KO MEFs, neither SB203580 nor PD169316 affected the abundance of oligonuclesomal fragments at any of the tested concentrations (Figure 22B and Figure 22C). The lack of effect of the two p38 inhibitors on basal apoptosis in Nrf2KO MEFs was striking, provided the well-established proapoptotic role of this stress-activated kinase in MEFs (Porras, Zuluaga et al. 2004). Thus we also studied if increased phosphorylation state of p38 was also present in lung and liver tissues from Nrf2KO mice, which had exhibited increased basal apoptosis levels (see Figure 13C and Figure 13D). Although p38 phosphorylation was increased in lung from Nrf2KO mice (Figure 22D), no changes were observed in liver (Figure 22E), which supports that the effect of Nrf2 deletion on the activation of p38 pathway is tissue specific, and probably unrelated to the alterations in basal apoptosis observed in cells lacking this antioxidant transcription factor.

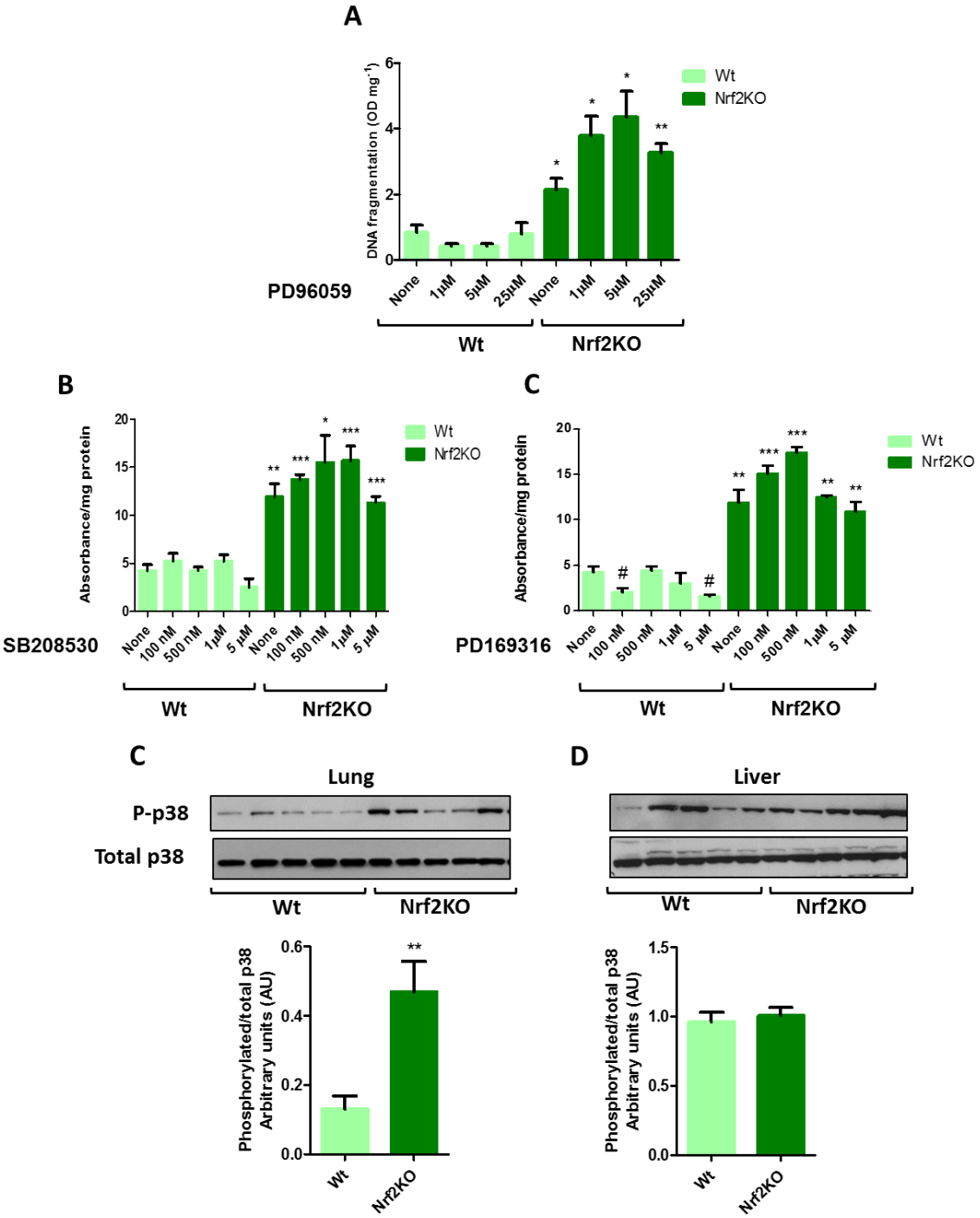

Figure 22: Role of ERK1/2 and p38 in apoptosis. A) ERK1/2 inhibitors: No effect on DNA fragmentation was observed when MEFs (either wild-type or Nrf2KO) were cultured in the presence of PD98059 at concentrations ranging from 1 to 25 µM. **B) p38 inhibitor, SB208530:** We found that the abundance of oligonuclesomal fragments was not affected by SB203580. **C) p38 inhibitor, PD169316:** PD169316 did not have any effect of DNA fragmentation at any of the concentrations in Nrf2KO. However, there was a decrease in apoptosis in wild-type MEFs at 100 nm and 5µM PD169316 concentration (#p<0.05). Significant differences between Wt and Nrf2KO MEFs were maintained in A) B) and C) (*p<0.05, **p<0.01 and ***p<0.001). Data are means ± SEM (n=4). **D) p38 phosphorylation state in lung:** p38 phosphorylation was increased in Nrf2KO

mice in comparison with their wild-type counterparts. **E) p38 phosphorylation state in liver:** No differences for p38 phosphorylation in liver were found between Nrf2KO and wild-type mice. Data are means ± SEM (n=5).

1.8. p38 regulates Bcl-2 levels in wild-type but not in Nrf2KO MEFs

It has been reported that p38 can regulate the levels and phosphorylation state of the anti-apoptotic protein Bcl-2 (De Chiara, Marcocci et al. 2006, Trouillas, Saucourt et al. 2008). Interestingly, it has been shown that the p38-mediated phosphorylation of Bcl-2 (Ser-87) inactivates the protein and decreases its antiapoptotic potential (De Chiara, Marcocci et al. 2006). Since, at least for some concentrations, pharmacological inhibition of p38 decreased DNA fragmentation in wild-type, but not in Nrf2KO MEFs, we aimed to see if there were differences in the effects of the two inhibitors on the levels of the anti-apoptotic protein Bcl-2 in MEFs from the two genotypes under study. For this purpose we treated the Wt and Nrf2KO MEFs with SB203580 or PD169316 at a concentration of 100 nM for 24 hours, and we then measured the levels of Bcl-2 and P-Bcl-2 by western blot followed by immunodetection with antibodies against total or Ser^{87} phosphorylated Bcl-2.

As already stated above, and confirming previous reports (Niture and Jaiswal 2012), Nrf2KO MEFs had lower levels of Bcl-2 when compared with wild-type cells. Of note, we found out that the inhibition of p38 significantly increased the Bcl-2 levels in wild-type MEFs whereas no changes were observed in the Nrf2KO after being treated with SB203580 or PD169316, which supports that p38-dependent regulation of Bcl-2 levels require Nrf2 (Figure 23A).

We next investigated if p38 also modulated Bcl-2 phosphorylation degree in our cellular system. In this case, we found that the phosphorylation degree of Bcl-2 was substantially elevated in Nrf2KO MEFs in comparison with the wild-type cells (Figure 23A). Although in principle this finding would agree with enhanced p38 kinase activity in the KOs, phosphorylation degree of Bcl-2 in Ser-87 was unchanged and remained elevated in Nrf2KO MEFs treated with p38 inhibitors. In contrast, pharmacological inhibition of p38 did affect the phosphorylation state of Bcl-2 in wild-type MEFs. As expected from a more prevalent role for p38 in regulating Bcl-2 phosphorylation in Nrf2-proficient cells, phosphorylation state of Ser-87 in Bcl-2 was significantly decreased in wild-type MEFs treated with SB203580 or PD169316. Since this treatment also resulted in increased total levels of Bcl-2 in wild-type MEFs (see above), our findings of a lower phosphorylation state of Bcl-2 (and hence more anti-apoptotic activity) further supports the existence of increased anti-apoptotic potential by p38 inhibition in Nrf2-proficient cells, which is blunted by Nrf2 deletion. The lack of effect of p38 inhibitors on Bcl-2 phosphorylation in Nrf2KO MEFs

argues in favor of the existence of additional kinases that regulate Bcl-2 phosphorylation, whose activity may be more prevalent in Nrf2-deficient cells.

To confirm the results obtained with p38 inhibitors on Bcl-2 levels, we also studied how the Nrf2 status affected the levels of Trx1, another well-established target of Nrf2 (Im, Lee et al. 2012) and how p38 inhibition affected Trx1 levels in wild-type and Nrf2KO MEFs. As found for Bcl-2 levels, Trx1 was also decreased in Nrf2KO compared with wild-type MEFs (Figure 23C). Remarkably, the effects of SB203580 or PD169316 on Trx1 and on Bcl-2 levels were essentially identical (compare Figure 23A and Figure 23C), and a substantial elevation of Trx1 by p38 inhibition was only observed in wild-type but not in Nrf2KO MEFs.

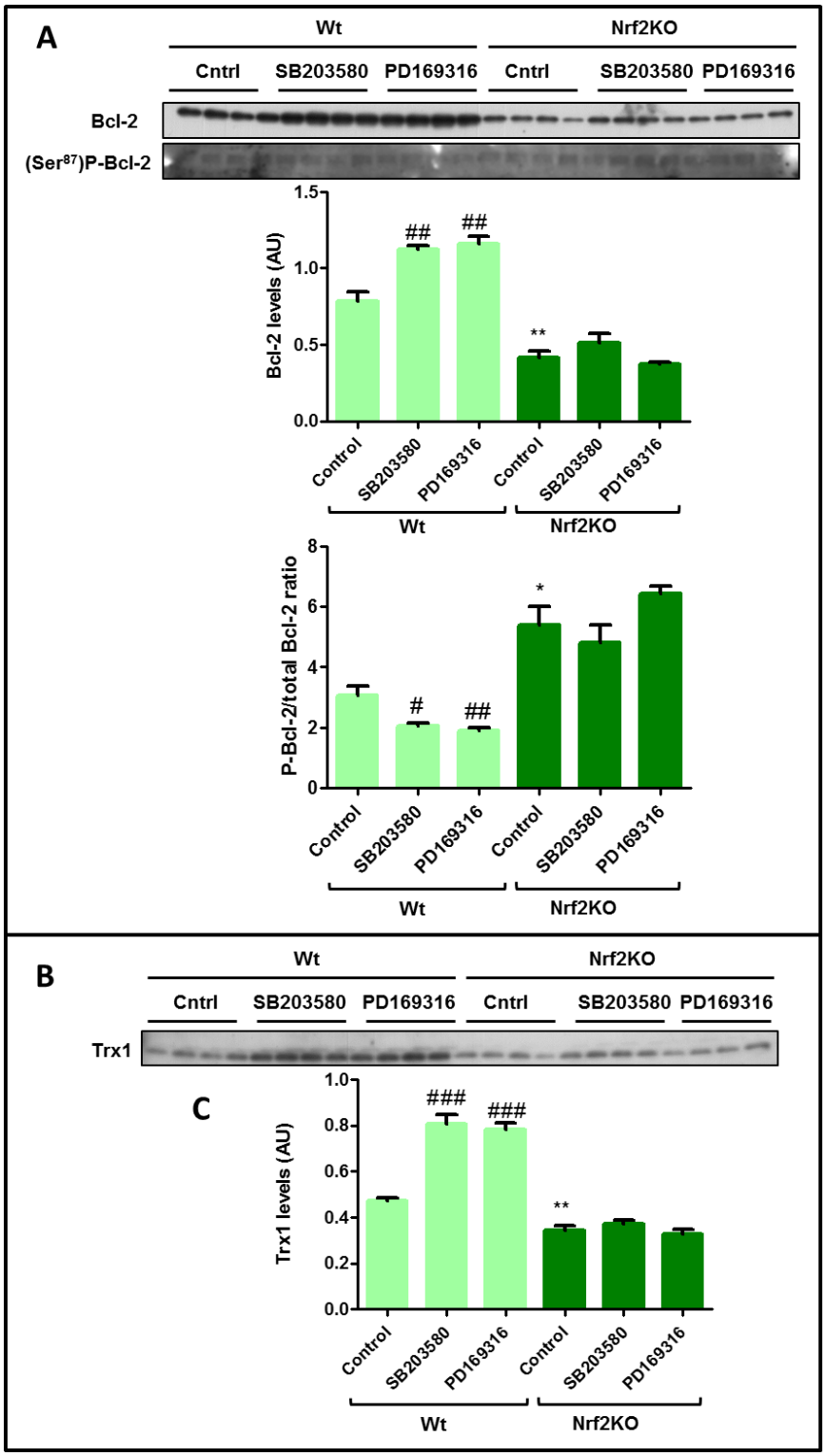

Figure 23: Bcl-2 modulation by p38. A) Bcl-2 and P-Bcl-2: Pharmacological inhibition of p38 with SB203580 or PD169316 significantly increased Bcl-2 levels and decreased its phosphorylation degree in wild-type MEFs (#p<0.05 and ##p<0.01). However, Bcl-2 levels were not restored by p38 inhibition in Nrf2MEFs and Bcl-2 remained equally low in these cells either in the absence or in the presence of p38 inhibitors (**p<0.01). **B) Trx1:** Trx1 was also decreased in Nrf2KO compared with wild-type MEFs (**p<0.01). A substantial elevation of Trx1 by p38 inhibition was only observed in wild-type but not in Nrf2KO MEFs (###p<0.001). Data are means ± SEM (n=4).

1.9. A role for monoamino oxidase B (MAO B) in the extent of basal apoptosis in Nrf2KO MEFs

Monoamine oxidase (MAO) is an enzyme located at the OMM that catalyzes the oxidative deamination of biogenic amines and is a quantitatively large source of H_2O_2. MAO contributes to an increase in the steady state concentrations of reactive species within both the mitochondrial matrix and cytosol leading to oxidative damage to the mitochondrial membrane and mitochondrial DNA (Cadenas and Davies 2000). MAO exists as two iso-forms, MAO-A and MAO-B, which are encoded by separate although highly homologous genes, and are independently regulated and distinguished on the basis of their substrate specificity and inhibitor affinity. Whereas MAO-A preferentially deaminates 5-hydroxytryptamine (serotonin), adrenaline and noradrenaline, and is irreversibly inhibited by clorgyline at low micromolar concentrations, MAO-B preferentially deaminates β-phenylethylamine and tele-methylhistamine and is inhibited by deprenyl (Sivasubramaniam, Finch et al. 2003). Most studies on MAOs are related with the central nervous system organs (CNS), where MAOs may participate in several psychiatric and neurogenerative disorders when expressed in an aberrant way. However, mRNAs and proteins of both MAOs show a widespread distribution in non-CNS tissues (Sivasubramaniam, Finch et al. 2003).

Interestingly, a previous massive transcriptomic study highlighted MAO-B as a potentially upregulated gene in Nrf2KO murine alveolar type II cells (Reddy, Kleeberger et al. 2007). Since MAO activity has been related with mitochondrial permeabilization, which can be abolished by inhibitors of both MAO-A and B in rat liver mitochondria (Marcocci, De Marchi et al. 2002), we tested the effect of chlorgyline and deprenyl, two well-characterized inhibitors of MAO-A and MAO-B respectively on basal DNA fragmentation in wild-type and Nrf2KO MEFs. In our hands, the MAO-A inhibitor chlorgyline was found toxic for MEFs at concentrations of 10μM and higher (data not shown). At 1μM, chlorgyline decreased DNA fragmentation in wild-type, but had no effect on Nrf2KO MEFs. Toxic effects of deprenyl were observed from a concentration of 100μM. However, at 1 and 10 μM this MAO-B inhibitor decreased DNA fragmentation both in wild-type and Nrf2KO MEFs in a concentration-dependent manner (Figure 24A).

Although it has been reported that MAO-A is the predominant MAO isoform in fibroblasts (Chen 2004), MAO-B polypeptide was easily demonstrated in western blots (Figure 24B) and showed higher abundance in Nrf2KO MEFs in relation to their wild-type counterparts. This is the first demonstration that deletion of Nrf2 upregulates MAO-B protein levels, which is fully in accordance with the previous identification of the MAO-B gene as

potentially upregulated in a transcriptomic study carried out with Nrf2KO cells (Reddy, Kleeberger et al. 2007).

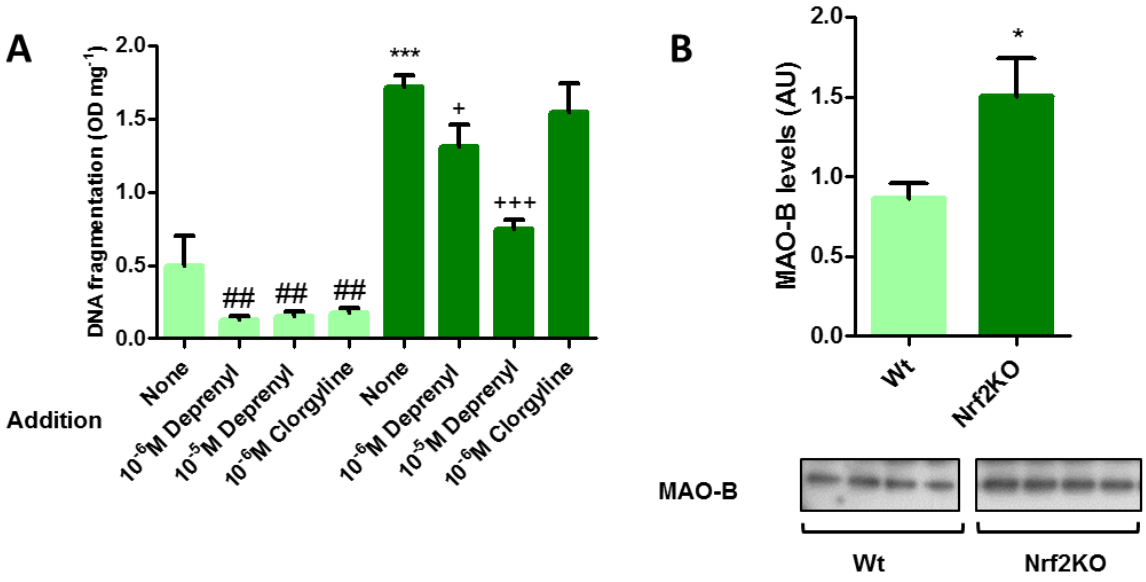

Figure 24: MAO inhibition. A) Effects of the MAO-B and MAO-A inhibitors deprenyl and chlorgyline: At 1μM, chlorgyline decreased DNA fragmentation in wild-type, but had no effect on Nrf2KO MEFs (##p<0.01). However, deprenyl inhibited DNA fragmentation both in wild-type and in Nrf2KO MEFs in a concentration-dependent manner (for wild-type ##p<0.01 and for Nrf2KO MEFs +p<0.05 and ++p<0.01). **B) MAO-B levels in wild-type and Nrf2KO MEFs:** MAO-B protein levels were higher in Nrf2KO than wild-type MEFs (*p<0.05). Data are means ± SEM (n=4).

1.10. Effect of XIAP inhibition in the viability of wild-type and Nrf2KO MEFs

Our results on apoptotic signaling alterations in Nrf2KO MEFs had pointed out towards the activation of a caspase-independent pathway through AIF. This interpretation was likely because of the lack of caspase-9 and -3 activation, despite significant mitochondrial permeabilization and release of cytochrome *c* to the cytosol in Nrf2KO MEFs which displayed a significant elevation in cytosolic oligonucleosomal fragments. Moreover, the levels of the other two protein components of the apoptosome, pro-caspase-9 and Apaf-1 were also significantly upregulated in Nrf2KO MEFs. Inhibitors of apoptotic proteases (IAPs) such as XIAP can avoid the engagement of mitochondrial apoptosis by inhibiting both caspase-9 and -3, and this anti-apoptotic activity is antagonized by pro-apoptotic factors such as Smac/DIABLO which are also released from the mitochondria (Chai, Du et al. 2000). Thus, we decided to study the cell viability responses of wild-type and Nrf2KO MEFs to the Smac/DIABLO mimetic and XIAP inhibitor BV6 (Ehrenschwender, Bittner et al. 2014). Cells were treated with BV-6 at 3.12 μM, 6.25 μM and 12.5 μM for 48 hours and thereafter a MTT assay was performed to assess for cell viability. As shown in Figure 25, the treatment with

BV-6 decreased viability of Nrf2KO MEFs from the lowest tested concentration in a concentration-dependent manner. However, wild-type MEFs treated with BV-6 did not show any effect on cell viability at any of the tested concentrations (Figure 25A). We also aimed to detect any variation in procaspase-3 processing by western blot and immunodetection. We observed no changes in procaspase-3 abundance in wild-type MEFs, either without treatment or after being treated with BV6 at 12.5μM. However, the abundance of procaspase-3 was significantly lower in Nrf2KO MEFs after being treated with the XIAP inhibitor (Figure 25B), which is indicative of enhanced proteolytic processing.

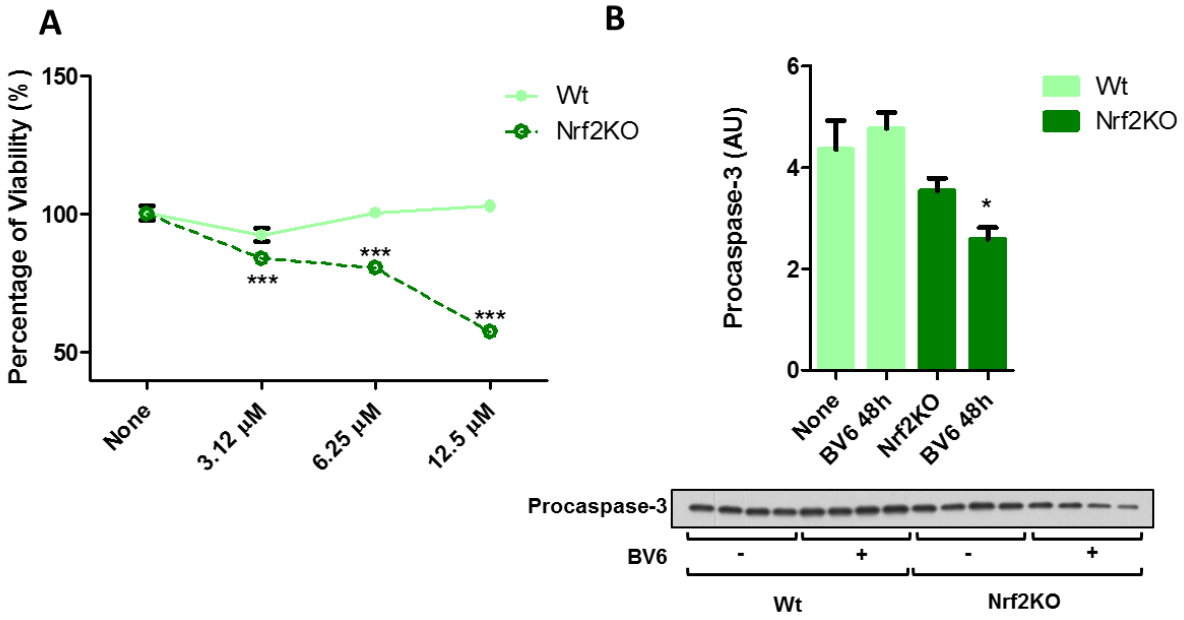

Figure 25: XIAP inhibition. A) Viability assay: Treatment with BV-6 decreased viability in Nrf2KO MEFs from the lowest tested concentration in a concentration-dependent manner (***p<0.001). However, wild-type MEFs treated with BV-6 did not show any effect on cell viability. **B) Procaspase-3 processing:** We observed no changes in wild-type MEFs either without treatment or after being treated with BV6 at 12.5μM. However, the abundance of procaspase-3 was significantly lower in Nrf2KO MEFs when treated with the XIAP inhibitor (*p<0.05). Data are means ± SEM (n=4).

1.11. Mitochondria show ultrastructural alterations in Nrf2KO MEFs

Previous preliminary results obtained in our group had indicated the existence of some ultrastructural alterations in mitochondria in Nrf2KO MEFs, measured as differences in the diameter of the mitochondria in the Nrf2KO MEF mitochondria. Here, we aimed to perform a more complete planimetric study to have more information about putative alterations in mitochondrial size due to genetic deletion of Nrf2. We have found that mitochondria are significantly smaller in Nrf2KO MEFs when compared to their wild-type counterparts.

However, circularity did not change. Also, we have confirmed the existence of a higher number of mitochondria per μm² of cytoplasm, and the portion of cytoplasm occupied by mitochondria was also higher.

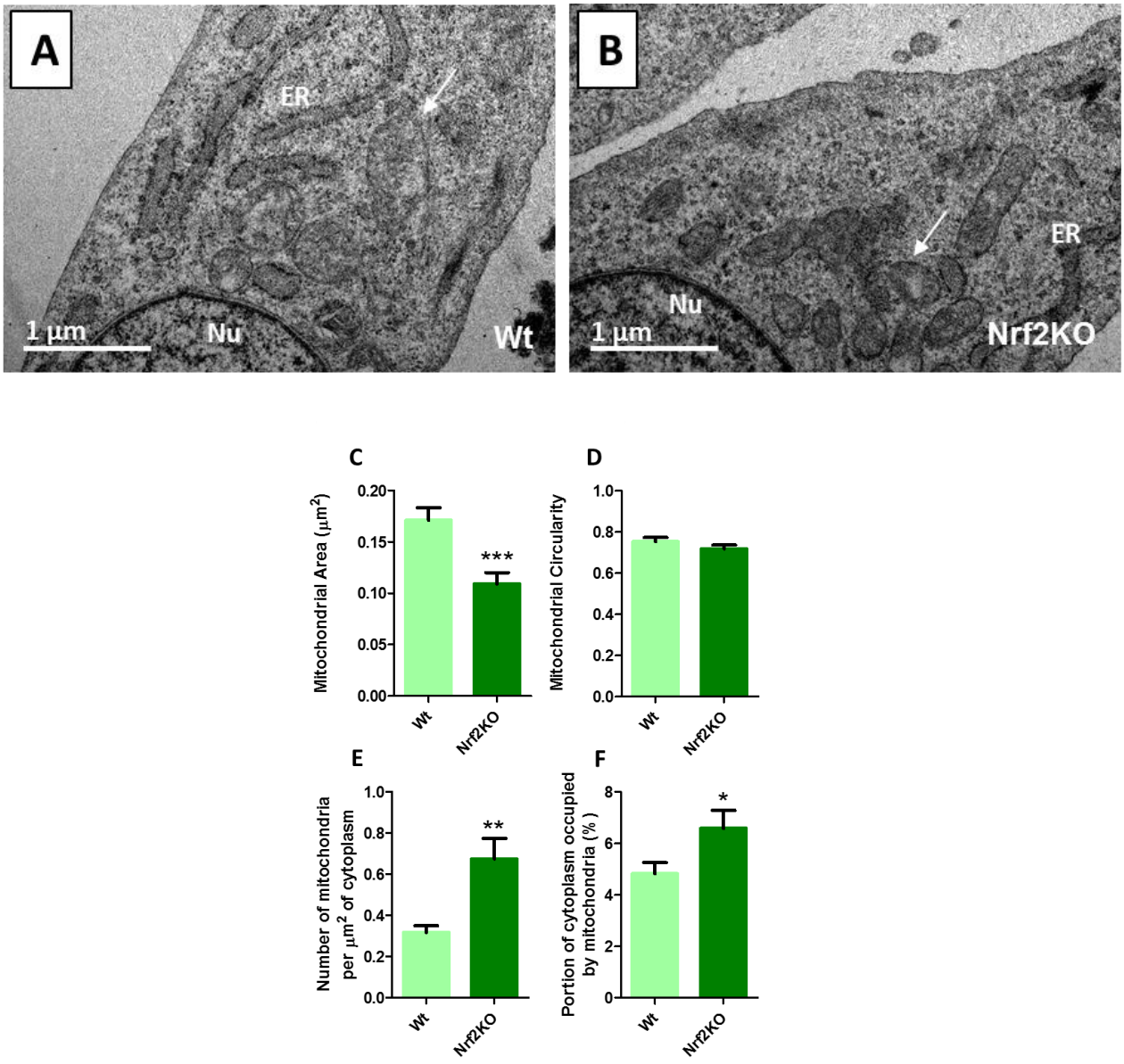

Figure 26: Ultrastructural alterations in Nrf2KO MEFs. A) Wild-type MEFs representative micrograph. B) Nrf2KO MEFs representative micrograph. C) Mitochondrial area: We have found that mitochondria are significantly smaller in Nrf2KO MEFs when compared to their wild-type counterparts (***p<0.001). **D) Mitochondrial circularity:** Circularity did not change among genotypes. **E) Number of mitochondria per area of cytoplasm:** There is a higher number of mitochondria per μm² of cytoplasm in Nrf2KO MEFs when compared to their wild-type counterparts (**p<0.01). **F) Portion of cytoplasm occupied by mitochondria:** The portion of cytoplasm occupied by mitochondria is also higher in Nrf2KO MEFs (*p<0.05).

1.12. Alterations of the master regulator of mitochondrial biogenesis PGC1α

The regulation of cellular and mitochondrial metabolism is controlled by complex networks. In recent years, the peroxisome proliferator-activated receptor γ coactivator 1 (PGC1) family of transcriptional coactivators has emerged as central regulators of metabolism. The PGC1 family consists of three members, namely PGC1α, PGC1β and the PGC related coactivator (PRC), which interact with transcription factors and nuclear receptors to exert their biological functions (Handschin and Spiegelman 2006). The most studied member of the PGC1 family is PGC1α, which is a positive regulator of mitochondrial biogenesis and respiration, adaptive thermogenesis, gluconeogenesis as well as many other metabolic processes (Handschin and Spiegelman 2006). Since genetic deletion of Nrf2 had produced alterations in several parameters related with mitochondrial morphology, we wanted to study if PGC1α levels were also altered in cells lacking Nrf2. When we measured the levels of PGC1α by western blot and immunodetection we found that Nrf2KO immortalized MEFs showed lower levels of PGC1α than their wild-type counterparts (Figure 27A).

1.13. Mitochondrial dynamics markers

Mitochondria fuse and divide in response to cell demands and environment. Alterations in mitochondrial dynamics underlie various diseases. As Nrf2 deletion resulted in alterations of the apoptotic process and mitochondria morphology, we aimed to study several markers of the mitochondrial dynamics processes of fusion and fission. For this purpose we measured by western blot and immunodetection the levels of three proteins involved in mitochondrial fusion: Mfn1, Mfn2 and Opa1, and two protein mediating fission events: Fis1 and Drp1.

Regarding the proteins involved in mitochondrial fusion, it was found that levels of Mfn2 were decreased in Nrf2KO in comparison with wild-type MEFs (Figure 27E). However, changes in Mfn1, the other fusion protein of the OMM, did not reach significant differences (Figure 27D). The IMM fusion marker Opa1 did not show significant differences either (Figure 27F). On the other hand, regarding fission markers neither Drp1 nor Fis1 levels were changed in Nrf2KO MEFs when compared to their wild-type counterparts (Figure 27B and Figure 27C).

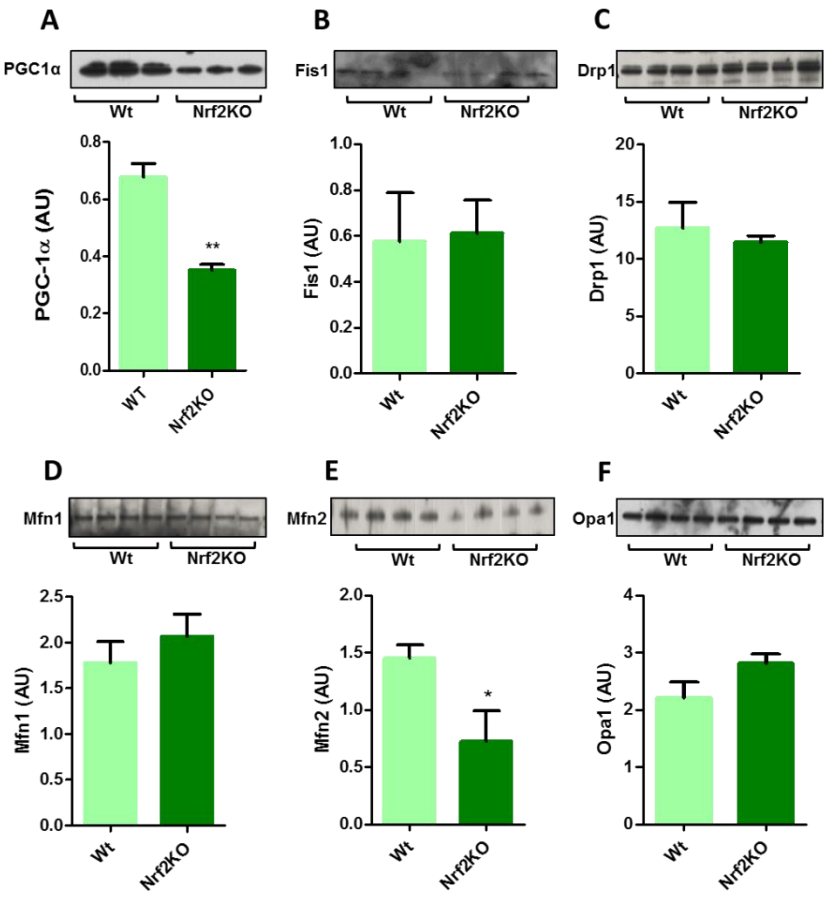

Figure 27: Mitochondrial biogenesis and dynamics markers. A) PGC1α: Nrf2KO immortalized MEFs showed a lower level of PGC1α than their wild-type counterparts (**p<0.01). Data are means ± SEM (n=3). **B) Fis1:** This fission protein did not show any changes between genotypes. **B) Drp1:** No changes were observed in Nrf2KO MEFs when compared to their wild-type counterparts. **C) Mfn1:** Mfn1 levels did not change among Nrf2KO and wild-type MEFs. **E) Mfn2:** The expression levels were decreased in Nrf2KO in comparison with wild-type MEFs (*p<0.05). **F) Opa1:** This protein, located at the IMM, did not show significant differences between genotypes. Data are means ± SEM (n=4).

2. MEFs lacking NAD(P)H:quinone oxidoreductase 1 (NQO1)

2.1. Alterations in life span and proliferation rates of NQO1KO MEFS

Previous unpublished results obtained in our group have shown differences in long-term culture between wild-type and NQO1KO MEFs. Wild-type MEFs grew steadily until passage 62 and presented a proliferation limit at 64 passages. However, NQO1KO MEFs had a proliferation limit that reached around 80 passages. In addition, proliferation rates, measuring as doubling rates, of NQO1KO cells seemed to be slightly higher when compared to the wild-type MEFs (Jodar 2010). First, we wanted to confirm the apparent growth advantage of NQO1KO MEFs by using the thymidine incorporation proliferation assay using radiolabeled thymidine.

We observed that the level of [^{3}H]-thymidine incorporated into DNA was significantly higher for immortalized NQO1KO MEFs in comparison with the wild-type cells, which confirmed our previous results for doubling rates (Figure 28).

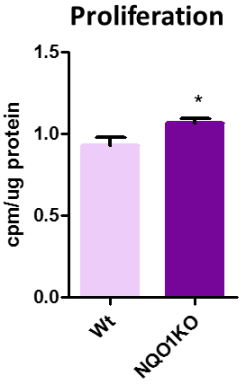

Figure 28: Proliferation assay. The levels of ^{3}H-thymidine incorporated into DNA was significantly higher for NQO1KO immortalized MEFs in comparison with their wild-type counterparts (*p<0.05). Data are means ± SEM (n=6).

Since cells growth results from the balance between proliferation and apoptosis, we next aimed to measure putative changes in apoptotic markers in NQO1KO MEFs.

2.2. Apoptotic Index

To elucidate the levels of basal apoptosis in the cell population, we carried out the measurement of cytosolic oligonucleosomal fragments abundance. In this determinations, we observed significant differences in the DNA fragmentation between the two genotypes that were compared, with NQO1KO MEFs showing a higher apoptotic index in comparison with their wild-type counterparts (Figure 29).

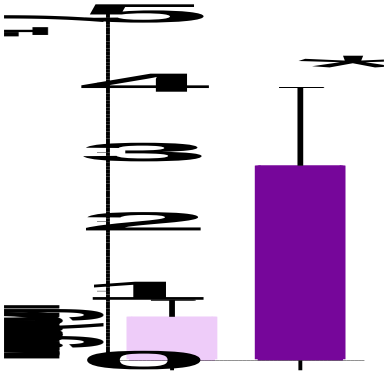

Figure 29: Apoptotic index. Levels of oligonucleosomal fragments were increased in the immortalized NQO1KO MEFs when compared to their wild-type counterparts (*p<0.05). Data are means ± SEM (n=6).

To try to understand the mechanism by which the NQO1KO cells have higher apoptotic levels we analyzed the levels of different parameters such as the Bcl-2 family proteins.

2.3. Phophorylated histone H2AX

Histone H2AX phosphorylated at serine 139 (γH2A.X) is a specific marker for the existence of a DNA DSBs (Takahashi, Mori et al. 2008). Since significant differences in DNA fragmentation had been observed between NQO1KO and wild-type MEFs, we also wanted to compare cells from these two genotypes for phosphorylated histone H2AX staining by immunofluorescence. Strikingly, we found no changes between both phenotypes for the intensity of fluorescence, measured as mean grey value at ImageJ (Figure 30A and Figure 30B), which is in contrast with increased DNA fragmentation in NQO1KO MEFs.

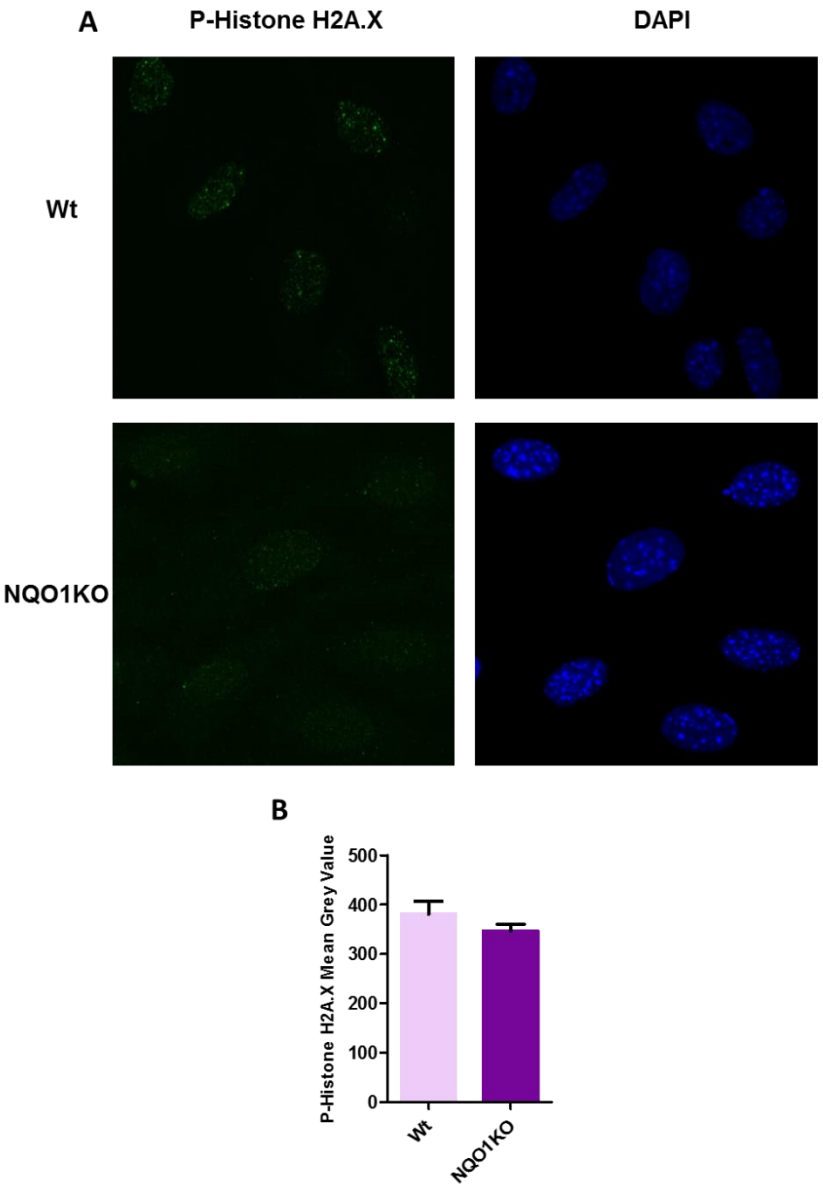

Figure 30: Phosphorylated H2A.X (Ser139). A) Representative images of DAPI and P-H2A.X staining. B) Quantification of fluorescence intensity: NQO1KO MEFs did not evidence changes in γH2A.X staining when compared to the wild-type MEFs. Data are means ± SEM (n=60).

2.4. Mitochondrial apoptosis signaling

In order to elucidate the mechanism by which higher levels of apoptosis were achieved in NQO1KO MEFs in comparison to their controls, we aimed to study several proteins from the Bcl-2 family, as the main regulators of mitochondrial apoptosis. We also measured the levels of proapoptotic proteins that are released to the cytosol after the permeabilization of the outer mitochondrial membrane.

2.4.1. Bcl-2 and Bax polypeptides

We analyzed two important members of the Bcl-2 family of proteins, the antiapoptotic factor Bcl-2 and the proapoptotic polypeptide Bax. In our hands, Bax levels did not change among wild-type and NQO1KO MEFs (Figure 31B). However, we observed a significant increase in the Bcl-2 levels in NQO1KO MEFs (Figure 31A). When we calculated the Bcl-2/Bax ratio, an indicator of the enhancement of the intrinsic apoptotic pathway, we observed this indicator was increased in NQO1KO MEFs (Figure 31C), which is indicative of anti-apoptotic signaling in cells lacking NQO1. This finding was surprising, provided the increase of DNA fragmentation in the same cells (see above).

2.4.2. Proapoptotic mitochondrial factors: cytochrome *c* and AIF

We compared the release of two different factors from the mitochondrial IMS to the cytosol (as is the case of cytochrome *c*) or to the nucleus (as is the case of AIF). In the case of the cytosolic levels of cytochrome *c*, an increase in the cytochrome c released to the cytosol was found in the NQO1KO in relation to the wild-type MEFs (Figure 31D). When nuclear AIF levels were tested, the NQO1KO cells also evidenced a higher nuclear translocation of AIF (Figure 31E).

2.4.3. Apaf-1 levels

We also evaluated how genetic deletion of NQO1 affected Apaf-1 levels. As indicated earlier, cytochrome *c* release is a crucial event of the intrinsic pathway, activating Apaf-1 to form the apoptosometogether with dATP and cytochrome *c*. Apoptosome recruits and activates caspase-9 and subsequently caspase-3 (Bratton and Salvesen 2010). In this case, we found that this pro-apoptotic adaptor protein did not change among wild-type and NQO1KO MEFs (Figure 31F).

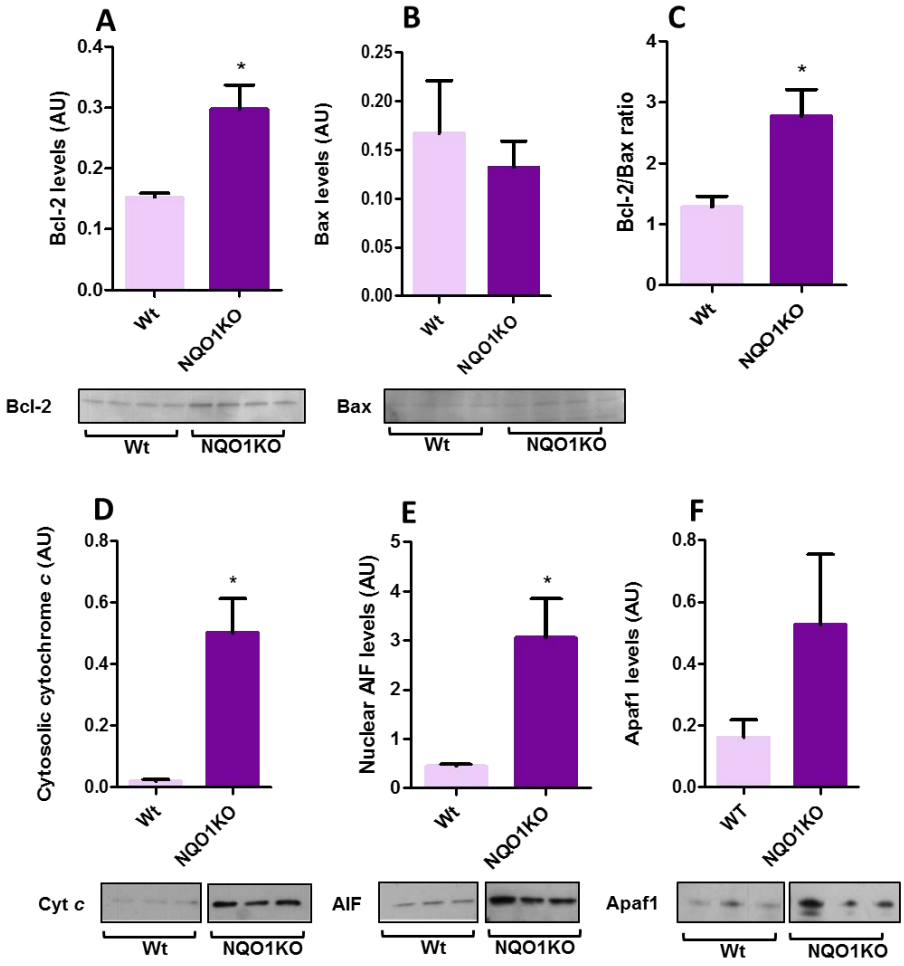

Figure 31: Mitochondrial pathway markers. A) Bcl-2 levels: We found out that the levels of Bcl-2 were increased in the NQO1KO immortalized MEFs if compared to their Wt counterparts (**p*<0.05). **B) Bax levels:** The levels of Bax polypeptide did not change in the NQO1KO MEFs when compared to their Wt counterparts. **C) Bcl-2/Bax ratio:** The Bcl-2/Bax ratio was increased in NQO1-null immortalized cells (**p*<0.05). Data are means ± SEM (n=4). **D) Cytosolic cytochrome *c*:** The abundance of cytochrome *c* in the cytosol was higher in NQO1KO compared to wild-type MEFs (**p*<0.05). **E) Nuclear AIF:** Levels of nuclear AIF were significantly elevated in NQO1-null cells (**p*<0.05). Data are means ± SEM (n=3). **F) Apaf-1 levels:** Changes in Apaf-1 levels in NQO1KO MEFs did not reach significant differences. Data are means ± SEM (n=3).

2.5. Mitochondrial potential and ROS production

A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria. This mitochondrial disruption includes changes in the mitochondrial membrane potential (ΔΨ), presumably due to the permeabilization of the OMM that allows the passage of ions and small molecules (Garrido, Galluzzi et al. 2006). We aimed to compare the ΔΨ of the wild-type and the NQO1KO MEFs and found that the ΔΨ of the overall population was significantly lower in the NQO1-null cells in relation with their wild-type counterparts (Figure 32A).

Also we aimed to quantify levels of intracellular ROS. For that purpose we used the fluorogenic probe DCFH-DA and found out that levels of intracellular peroxide was lower in NQO1KO MEFs (Figure 32B).

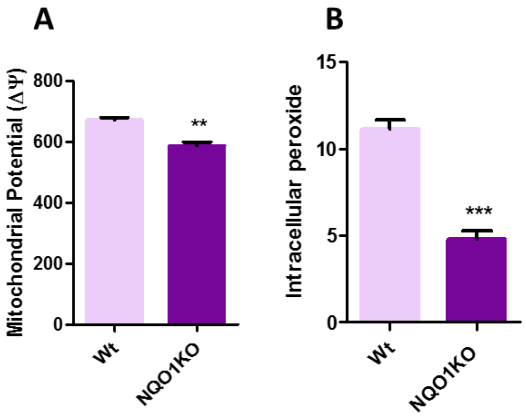

Figure 32: Mitochondrial potential and intracellular peroxide levels. A) Mitochondrial potential: The mitochondrial potential was lower in the NQO1KO MEFs in relation to the wild-type cells (**p<0.01). **B) Intracellular peroxide levels:** The levels of intracellular peroxide are lower in NQO1KO MEFs when compared to their wild-type counterparts (***p<0.001). Data are means ± SEM (n=3).

2.6. Procaspases, activated caspases, Bid, tBid and XIAP

With the purpose of elucidate the apoptotic pathways involved in apoptosis engagement in NQO1KO cells, we next analyzed how deletion of NQO1 affected different caspase activities. We measured the activity of caspase-8, with is activated by proteolysis *via* the extrinsic pathway, caspase-9, a key caspase in the mitochondrial or intrinsic pathway apoptosis, and caspase-3, the most representative executioner caspase (Boatright and Salvesen 2003, Bratton and Salvesen 2010).

Caspase-9 activity showed no changes when comparing wild-type and NQO1KO MEFs (Figure 33A). The same observation was made for the executioner caspase-3 enzymatic activity (Figure 33B). However, caspase-8 evidenced a significant increase in the NQO1KO MEFs when compared to their wild-type counterparts (Figure 33C).

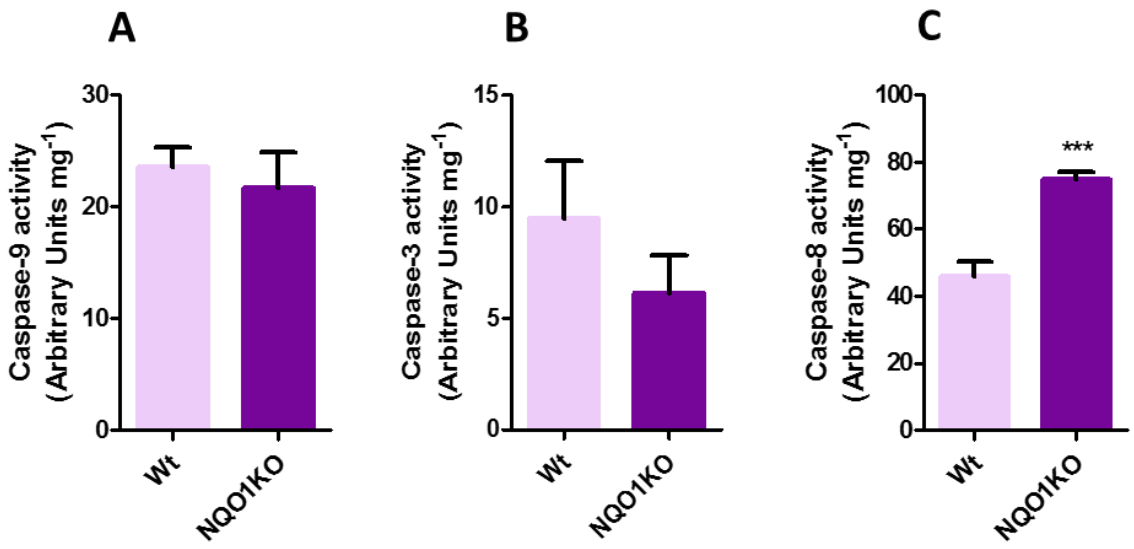

Figure 33: Caspase activities. A) Caspase-9 activity: Proteolytic activity of caspase-9 did not show changes among NQO1KO and wild-type MEFs. **B) Caspase-3 activity:** Enzymatic activity of caspase-3 did not change in NQO1KO cells in comparison to their wild-type counterparts. **C) Caspase-8 activity:** when we analyzed the enzymatic activity of caspase-8, a significant increase was observed for NQO1KO cells compared with their wild-type counterparts (***$p< 0.001$). Data are means ± SEM (n=5).

To confirm the results that we obtained using the enzymatic caspase assay, caspase activation was also studied by western blot determination of pro-caspases and their corresponding cleaved activated caspases.

The level of procaspase-9 was substantially increased in NQO1KO MEFs (Figure 34A), procaspase-3 was also more abundant in NQO1KO MEFs (Figure 34B) whereas procaspase-8 was slightly decreased (Figure 34C).

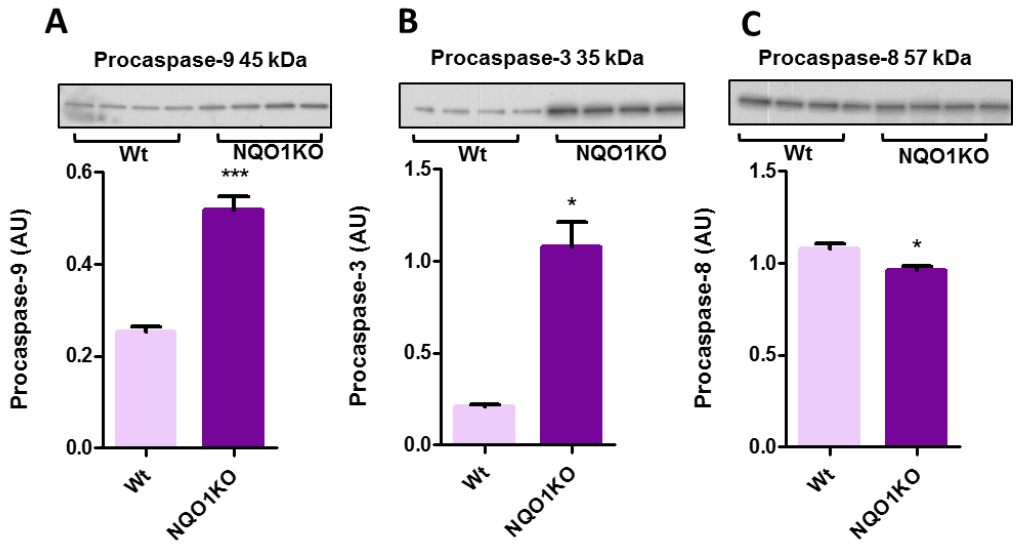

Figure 34: Procaspase levels. A) Procaspase-9: The level of procaspase-9 was substantially increased in NQO1KO MEFs (***p< 0.001). **B) Procaspase-3:** Levels of procaspase-3 were increased in NQO1KO MEFs (*p<0.05). **C) Procaspase-8:** Procaspase-8 was slightly decreased in NQO1-null MEFs (*p< 0.05).

In relation to the cleaved activated caspases, we observed a trend towards an increase of the activated 37/39 kDa fragments of caspase-9 in NQO1KO MEFs (Figure 35A). There was also an activation of caspase 3 17kDa fragment, but 19KDa cleaved did not reach significant differences among NQO1KO and wild-type MEFs (Figure 35B and Figure 35C). Also, the extrinsic pathway marker caspase-8 showed a dramatic increase of the 24/26 kDa and 18 kDa cleaved activated caspase-8 fragments (Figure 35D and Figure 35E).

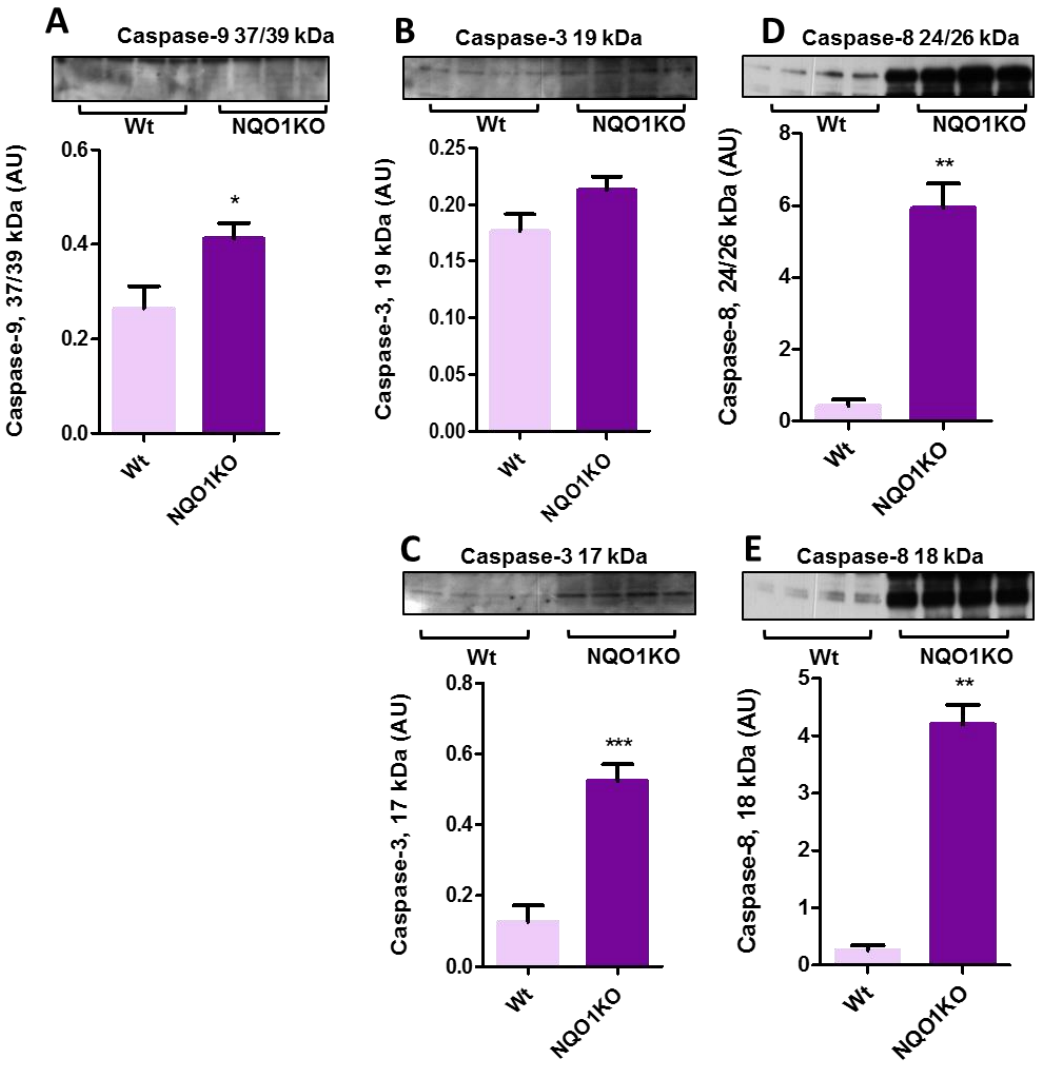

Figure 35: Cleaved caspase levels. A) Active caspase-9: We have observed an increase in the 37/39 kDa activated fragments of caspase-9 in NQO1KO MEFs (*p<0.05). **B) Active caspase-3:** Differences in 19KDa fragment did not reach significant levels. **C) Active caspase-3:** Higher abundance of 17 kDa fragment was found in NQO1KO MEFs (***p< 0.001). **D) and E) cleaved caspase-8:** Caspase-8 showed an increased in 24/26 kDa and 18 kDa activated caspase-8 fragments (**p< 0.01). Data are means ± SEM (n=4).

Cleavage of Bid by caspase 8 to generate tBid is the link between extrinsic apoptotic pathway and activation of mitochondrial apoptosis in type II cells, like MEFs (Luo, Budihardjo et al. 1998, Kantari and Walczak 2011) . Bid heterodimerizes with the anti-apoptotic protein Bcl-2 and with the pro-apoptotic protein Bax modulating their effects (Luo, Budihardjo et al. 1998). Since cleaved activated caspase-8 was significantly increased in NQO1KO MEFs we wanted to elucidate whether or not activation of caspase-8 played a prominent role in regulating basal apoptosis in MEFs lacking NQO1. To accomplish this objective we performed two different analyses: (i). to measure Bid and tBid levels in wild-type and NQO1KO MEFs, and (ii). to test the effect of Z-IETD-FMK, an irreversible and non-

toxic caspase-8 inhibitor, on apoptotic DNA fragmentation. Levels of full-length Bid were higher in NQO1KO MEFs when compared to their wild-type counterparts (Figure 36A). However, the cleaved form of Bid (tBid) did not change in the NQO1KO MEFs despite their increased levels of cleaved activated caspase-8. For the caspase-8 inhibition assay, cells were preincubated for 24 hours in the presence Z-IETD-FMK at several concentrations ranging from 5 to 25 μM. After preincubation with the caspase-8 inhibitor, the quantification of oligonucleosomal fragments levels by ELISA was performed. As shown in Figure 36B, we observed no effect on the abundance of cytosolic oligonucleosomal fragments and thus, basal apoptosis levels remained equally increased in the KOs, both in the absence and in the presence of the caspase-8 inhibitor at any of the tested concentrations.

One of the main IAPs is the X-chromosome linked Inhibitor of Apoptotic Proteases (XIAP), known to behave as a potent inhibitor of caspase-9 and effector caspases (Jost, Grabow et al. 2009). Thus, we also compared XIAP levels in wild-type and NQO1KO MEFs and found no change in XIAP protein levels among cells from the two genotypes (Figure 36C).

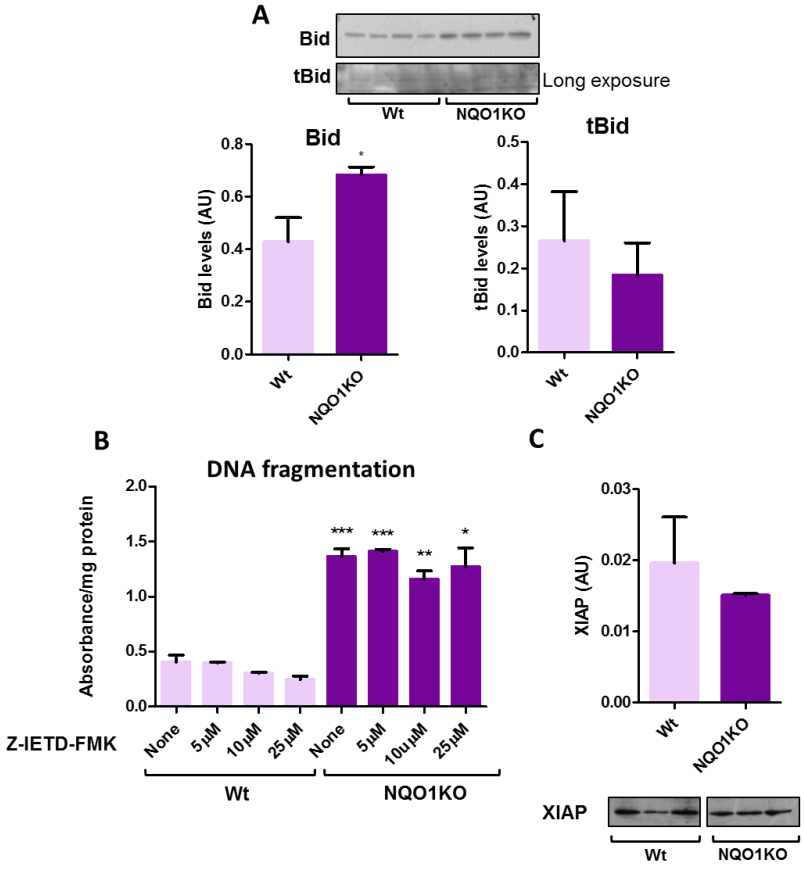

Figure 36: Role of caspase-8 activation and XIAP levels in apoptosis activation in NQO1KO MEFs. **A) Bid and tBid:** Levels of full-length Bid were higher in NQO1KO MEFs in relation to their wild-type

counterparts (*p<0.05). However, the abundance of tBid did not change among cells of the two genotypes. Data are means ± SEM (n=4). **B) Caspase-8 inhibitor treatment:** Treatment with caspase-8 inhibitor did not have any effect on DNA fragmentation and basal apoptosis levels remained equally increased in the NQO1KOs when compared with wild-type MEFs, both in the absence and in the presence of the caspase-8 inhibitor at any of the tested concentrations (*p<0.05, **p<0.01, ***p<0.001). Data are means ± SEM (n=4). **C) XIAP levels:** XIAP did not change significantly among NQO1KO and wild-type MEFs. Data are means ± SEM (n=3).

2.7. Nrf2 expression in NQO1KO MEFs and liver from NQO1KO mice

In the search for the mechanisms that could mediate longer life span and growth rate of NQO1KO MEFs, despite their higher mitochondrial permeabilization and caspase activation, we hypothesized that the lack of NQO1 could activate a retrograde signal that could boost Nrf2-dependent signaling to compensate for the loss of NQO1 functions. Activation of this process would result in a hormetic response leading to increased Nrf2 nuclear translocation and transcription of cytoprotective genes. In support for this idea, although NQO1KO MEFs indeed contained dysfunctional mitochondria which released proapoptotic factors as cytochrome *c* and AIF, the increase of Bcl-2 levels and Bcl-2/Bax ratio indicated the activation of anti-apoptotic signaling that was consistent with higher Nrf2-mediated gene expression. Thus, we measured Nrf2 levels in the cytosolic and nuclear compartments of wild-type and NQO1KO MEFs (*in vitro* cellular system), and also in fractions prepared from liver samples obtained from wild-type and NQO1KO mice (*in vivo* system). Both *in vivo* and *in vitro* measurements were performed under basal conditions and after a 6 hour treatment with tBHQ, a well-established Nrf2 inducer (Dinkova-Kostova and Wang 2011).

Nrf2 nuclear translocation was higher in NQO1KO MEFs under both basal and induced conditions (Figure 37A) and similar results were obtained for liver of NQO1KO mice (Figure 37B), which supports our hypothesis about the existence of a hormetic, Nrf2-dependent response in cells lacking NQO1.

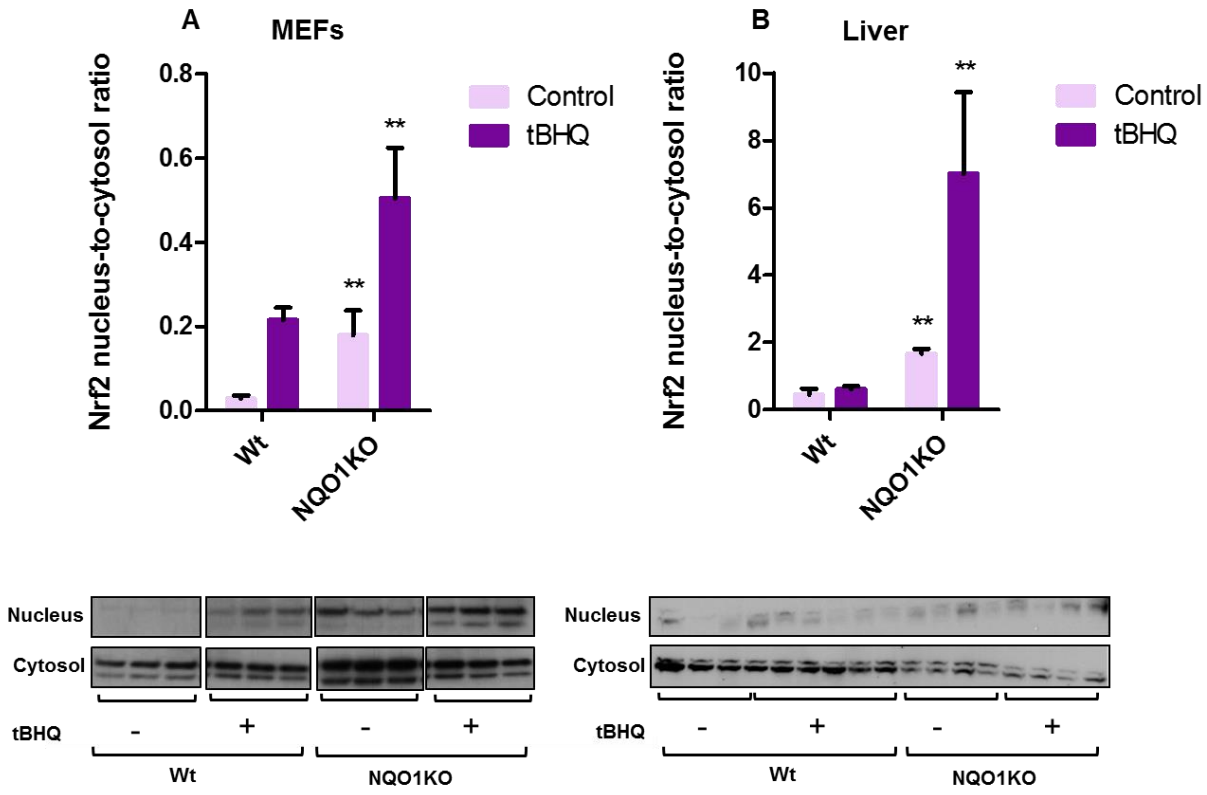

Figure 37: Nrf2 levels in cytosolic and nuclear fractions isolated from wild-type and NQO1KO MEFs and liver. A) MEFs: The nuclear-to-cytosol ratio, indicative for the nuclear Nrf2 translocation was higher in NQO1KO MEFs under both basal and tBHQ-induced conditions (**p<0.01). Data are means ± SEM (n=3). **B) Liver:** In this organ the values of the Nrf2 nucleus-to-cytosol ratio was also higher for NQO1KO mice both in basal and tBHQ-induced conditions (**p<0.01). Data are means ± SEM (minimum n=3).

To confirm that Nrf2-dependent signaling was stimulated in cells lacking NQO1 we also studied how the NQO1 status affected the levels of Trx1, a well-established target of Nrf2 (Im, Lee et al. 2012). We found that, consistently with the higher Nrf2 nucleus translocation, NQO1KO MEFs also exhibited higher levels of Trx1 in comparison to their wild-type counterparts (Figure 38A). The autophagy marker p62 is known to be involved in Nrf2 stabilization by interacting with Keap1 (Komatsu, Kurokawa et al. 2010). In the case of the levels of p62, we observed a trend towards an increase of p62 in total extracts of NQO1KO MEFs when compared with their wild-type counterparts (Figure 38B).

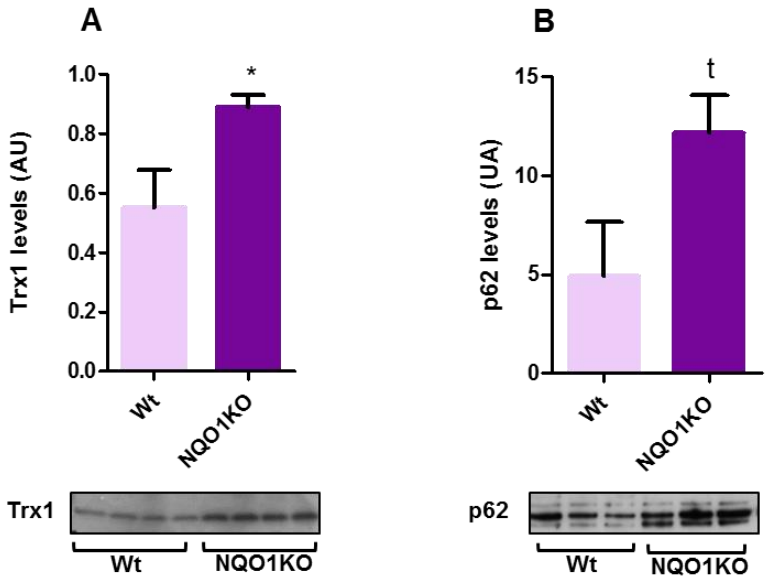

Figure 38: Trx1 and p62 levels in wild-type and NQO1KO MEFs. A) Trx1: Levels of Trx1 were higher in NQO1KO than their wild-type MEFs (**p*<0.05). Data are means ± SEM (n=4). **B) p62:** Our measurements evidenced a trend towards an increase of p62 levels with the two-tailed t-test in the NQO1KO MEFs (p=0.09), that was significant with the one-tailed t-test. Data are means ± SEM (n=3).

2.8. Alterations of MAPK and Akt pathways in NQO1KO MEFs

To further elucidate the mechanisms underlying the higher growth rates of NQO1KO immortalized MEFs we also analyzed by western blot and immunodetection the protein levels and activation of several MAPKs, Akt and a downstream Akt element, the glucose transporter Glut1 (Figure 39A).

Regarding p38, we found a dramatic decrease in the activated form of this stress-activated kinase in immortalized NQO1KO MEFs (Figure 39B). For JNK1, another MAPK activated under stress conditions, we also found a decrease in its activated form in NQOKO MEFs in comparison with the wild-type controls (Figure 39C). In relation with the determination of ERK1/2 our results showed no changes among genotypes (Figure 39D).

Also we wanted to quantify the levels of activated Akt, a protein involved in a pathway related to proliferation and survival, which protects cells against apoptosis (Altomare and Khaled 2012). We found that levels of phospho-Akt were higher in the NQO1KO MEFs in comparison to their wild-type counterparts (Figure 39E).

Related to this increase in phospho-Akt is the increase of the glucose transporter Glut1. Akt promotes cell proliferation among different pathways. One of them is the regulation of Glut1 activity and trafficking (Wieman, Wofford et al. 2007). In the case of this glucose transporter, its abundance was higher in NQO1KO MEFs (Figure 39F).

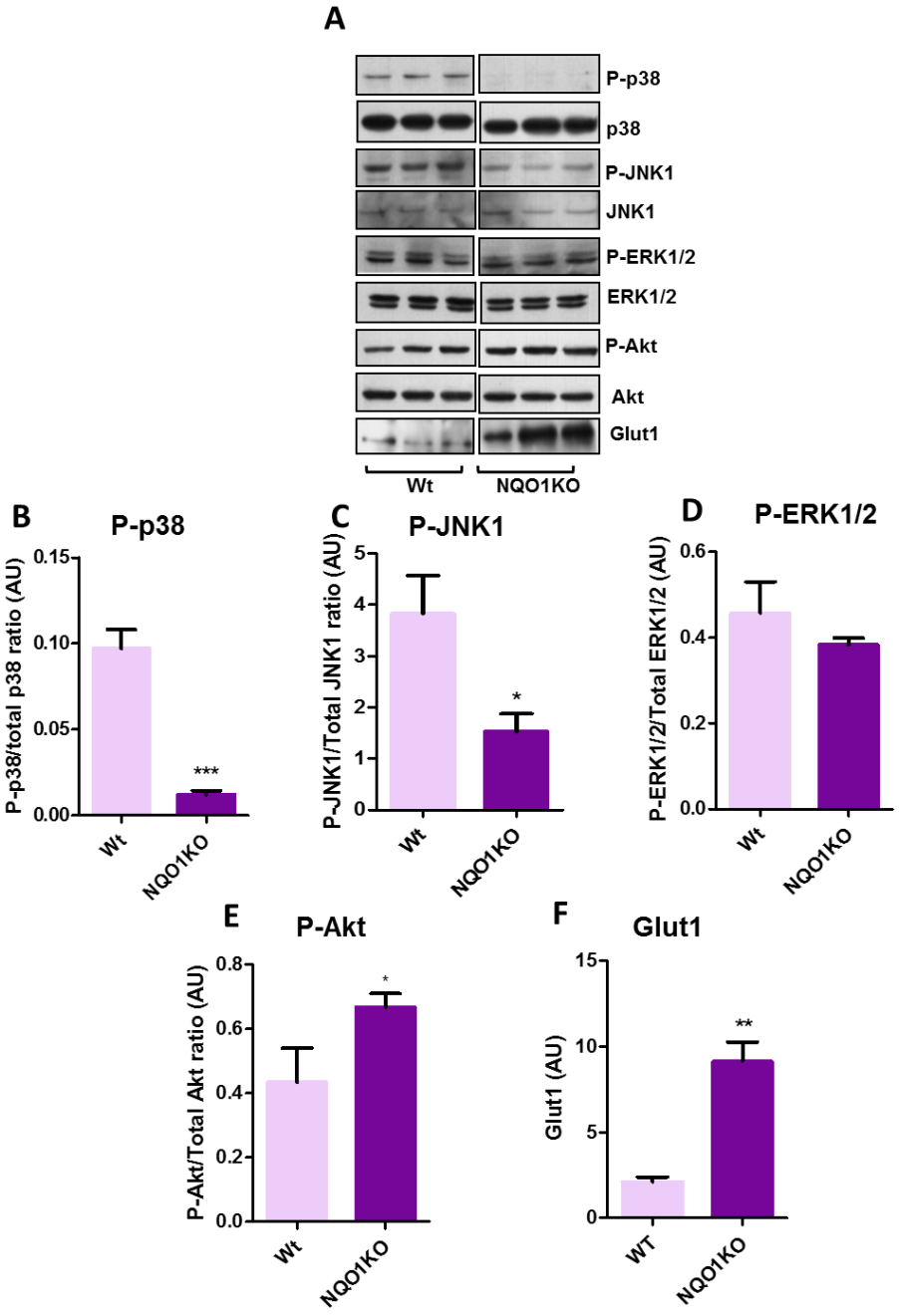

Figure 39: Alterations of MAPK and Akt pathways in NQO1KO MEFs. A) Western blost showing phosphorylated and total levels of MAPK, Akt and Glut1. B) p38 activation: Phosphorylation of p38 was decreased in NQO1KO MEFs in relation with their wild-type counterparts (***p<0.001). **C) JNK1 activation:** For JNK1 we also found a significant decrease in the NQO1KO cells (*p<0.05). **D) ERK1/2 activation:** The results showed no differences among wild-type and NQO1KO MEFs. **E) Akt activation:** Levels of phospho-Akt were higher in the NQO1KO MEFs in comparison to their wild-type counterparts (*p<0.05). **F) Glut1 levels:** This glucose transporter showed a higher abundance in NQO1KO MEFs (**p<0.01). Data are means ± SEM (n=3).

2.9. Mitochondrial ultrastructural alterations in NQO1KO MEFs

Previous preliminary results obtained in our group had showen certain ultrastructural alterations in mitochondria in NQO1KO MEFs, measured as differences in the diameter of the mitochondria in the NQO1KO MEFs. In this work, we aimed to perform a more complete planimetric study to have more information about putative alterations in mitochondrial size due to genetic deletion of NQO1. Firstly, we have found that mitochondria are significantly smaller in NQO1KO MEFs when compared to their wild-type counterparts (Figure 40C). Regarding circularity, data did not change among NQO1KO and wild-type MEFs (Figure 40D). In addition, we have confirmed preliminary results evidencing the existence of a higher number of mitochondria per μm^2 of cytoplasm (Figure 40E), and also the increase of the portion of cytoplasm occupied by mitochondria (Figure 40F).

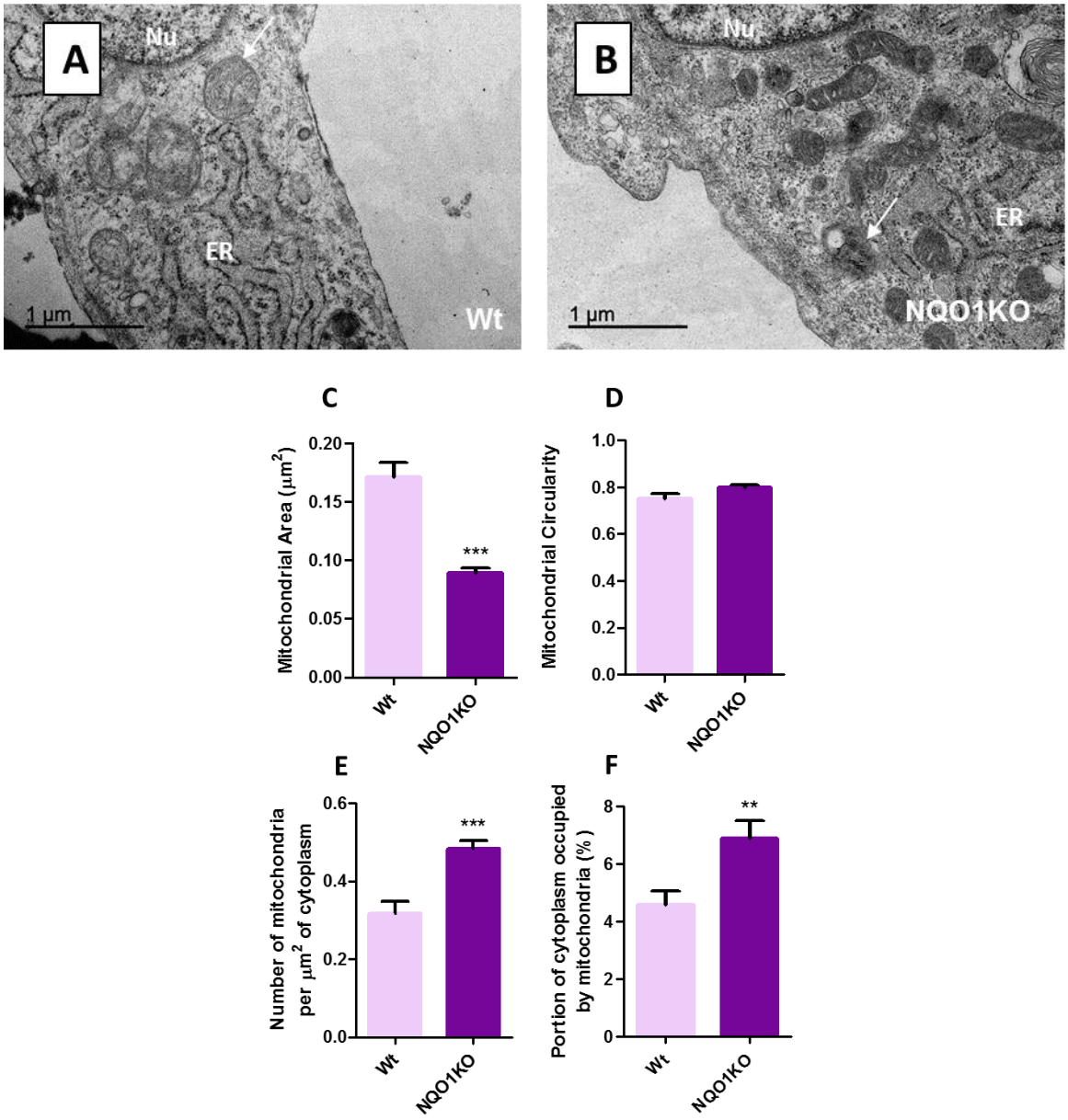

Figure 40: Ultrastructural alterations in NQO1KO MEFs. A) Wild-type MEFs representative micrograph. B) NQO1KO MEFs representative micrograph. C) Mitochondrial area: We have found that mitochondria are significantly smaller in NQO1KO MEFs when compared to their wild-type counterparts (***p<0.001). **D) Mitochondrial circularity:** Circularity did not change among genotypes. **E) Number of mitochondria per area of cytoplasm:** The number of mitochondria per μm² of cytoplasm was higher in NQO1KO MEFs when compared to their wild-type counterparts (***p<0.001). **F) Portion of cytoplasm occupied by mitochondria:** The portion of cytoplasm occupied by mitochondria is also higher in Nrf2KO MEFs (**p<0.01).

2.10. Effect of NQO1 deletion on mitochondrial biogenesis marker PGC1α

We next aimed to quantify levels of the positive regulator of mitochondrial biogenesis, PGC1α (Handschin and Spiegelman 2006). When we measured by western blot and immunodetection the levels of PGC1α we found that immortalized NQO1KO MEFs showed no differences with the wild-type cells (Figure 41A).

2.11. Mitochondrial dynamics markers

To gain new insights about the effects of NQO1 deletion on mitochondrial dynamics, we performed western blot and immunodetection to measure the levels of several mitochondrial dynamics regulators: the fusion proteins Mfn1, Mfn2 and Opa1, and the fission markers Fis1 and Drp1 in both NQO1KO and wild-type MEFs. Regarding the fusion protein Mfn2, it was found that its expression was significantly decreased in NQO1KO cells in comparison to their wild-type counterparts (Figure 41E) although we did not find significant changes in Mfn1 (Figure 41D). On the other hand, Opa1 was also significantly decreased in NQO1KO in comparison with the wild-type MEFs (Figure 41F). In relation to mitochondrial fission markers, the proteins which are involved in this process seem not to be altered because neither Fis1 (Figure 41B) nor Drp1 (Figure 41C) levels varied in NQO1KO MEFs when compared to the wild-type controls.

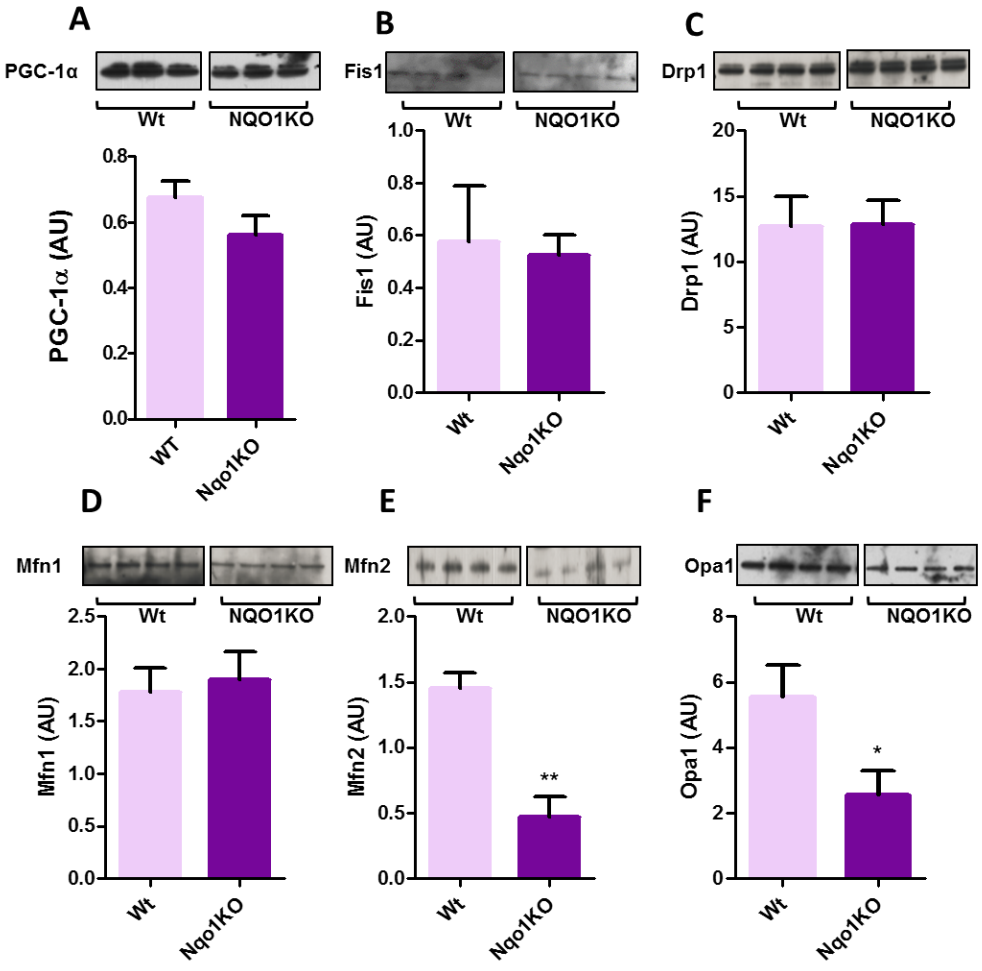

Figure 41: Mitochondrial biogenesis and dynamics markers. A) PGC1α: Immortalized NQO1KO and wild-type MEFs showed no changes in PGC1α levels. Data are means ± SEM (n=3). **B) Fis1:** This fission protein did not show any change among cells from the two genotypes. **C) Drp1:** No changes were observed in NQO1KO MEFs when compared to their wild-type counterparts. **D) Mfn1:** Mfn1 levels did not change among Nrf2KO and wild-type MEFs. **E) Mfn2:** Levels of Mfn2 were significantly decreased in NQO1KO MEFs in comparison to their wild-type counterparts (**p<0.01). **F) Opa1:** Levels of this fusion protein were also decreased in NQO1KO cells (*p<0.05). Data are means ± SEM (n=4).

2.12. Sox2: a stem cell marker in NQO1KO mice

Sox2 is an essential protein needed to maintain pluripotency in the embryonic cells (Boumahdi, Driessens et al. 2014). Sox2 was measured in liver and brain from wild-type and NQO1KO mice. As a positive control we used embryonic stem (ES) cells. As expected, the highest levels of Sox2 were found in ES cells. However, higher levels of Sox2 were also observed in the NQO1KO animals (both in liver and brain) in comparison to the wild-type counterparts (two-way ANOVA) (Figure 42).

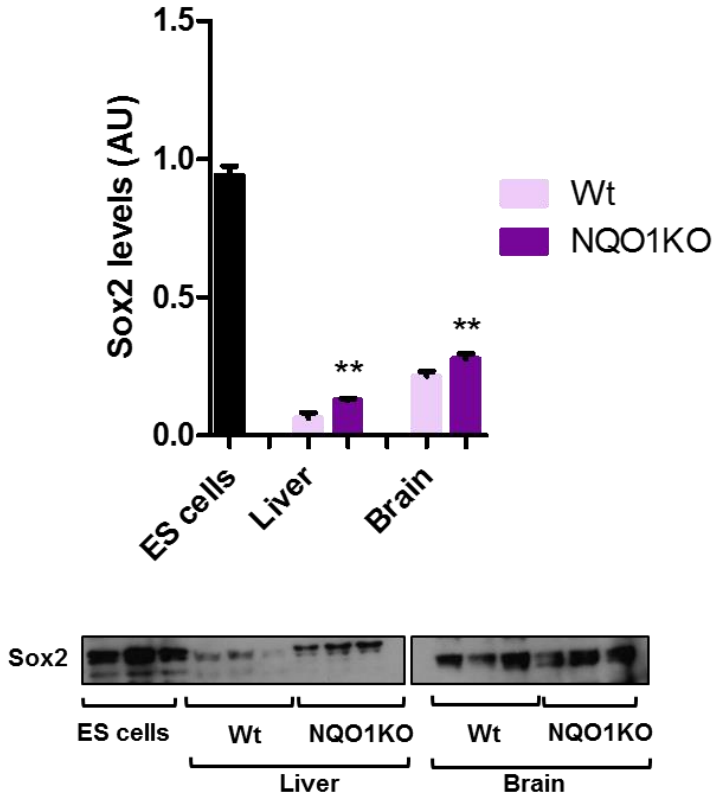

Figure 42: Sox2 in NQO1KO liver and Brain. The pluripotency marker was significantly higher both in liver and brain of NQO1KO mice (**p<0.01) (two-way ANOVA).

2.13. Wound healing assay

The NQO1KO cells seem to have alterations in mitochondrial morphology and function that promote mitochondrial permeabilization and apoptosis. However, we have also observed lower levels of activated p38 and JNK1 in these cells, as well as higher levels of proliferation markers as phosphor-Akt and Nrf2. The glucose transporter Glut1 also showed higher levels in the NQO1KO MEFs. Taken into account the proliferative characteristics of these cells, we aimed to assess the migration capacity of NQO1KO MEFs. The wound healing *in vitro* assay mimics the cell migration in the process of wound healing in tissues *in vivo*. In order to estimate the migration properties of this cells in relation to their wild-type counterparts, we measured the healing process at time zero and after 8 hours. We found out that, after being cultured for this time, the wound was almost 100% healed in the NQO1KO MEFs whereas only a 30% of healing was reached in the wild-type MEFs (Figure 43A and Figure 43B).

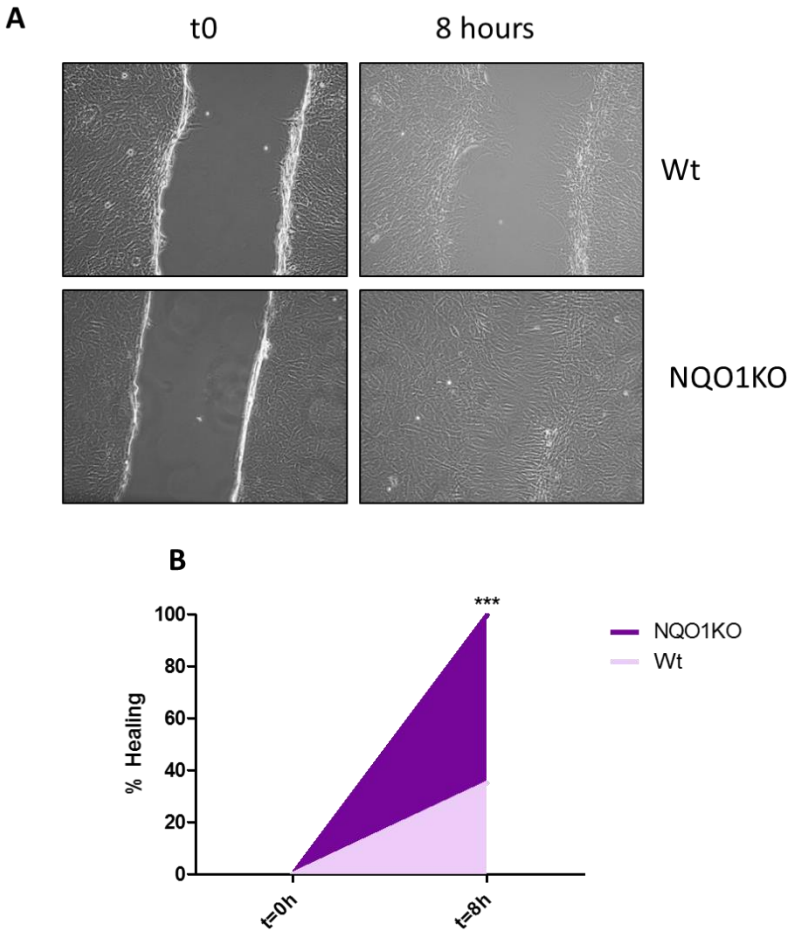

Figure 43: Wound healing assay. A) Representative micrographs. B) Healing percentage: We found out that, after 8 hours culture, the wound was almost 100% healed in the NQO1KO MEFs whereas only a 30% of healing was reached in the wild-type MEFs (***p<0.001).

2.14. Cell injection and tumor induction in nude mice

An *in vivo* assay to evaluate pluripotency and determine the tumorigenic potential of the NQO1KO MEFs in comparison to the wild-type cells is tumor induction by cell injection, an *in vivo* assay for teratoma formation. Positive controls for tumor formation were carried out by injecting B10F16 cells from mouse skin melanoma. Besides the positive control, wild-type and NQO1KO MEFs were also injected into nude mice. The total load of cells was about 1.5 million per injected animal. Animals were sacrificed 26 days after the injections. As depicted in Figure 44A, when the mouse skin melanoma cells were injected in nude mice, they generated tumors in 100% of the animals that had been injected with this cell type (n=12). With regards to injections with MEFs, the results were very consistent: we found that the 12 out of the 13 nude mice injected with the NQO1KO generated a tumor in the place were cells had been injected, whereas the wild-type MEFs did not generate tumors when injected into the nude mice.

Tumors were isolated, fixed and processed for histological observation. After haematoxylin and eosin staining of the tumor sections we found out that the tumors were teratomas composed by a number of mesoderm-derived tissues. We were able to identify tissues like muscular tissue (Figure 44D), white adipose tissue (Figure 44B) or brown adipose tissue in the teratoma sections (Figure 44C). Also in certain areas of the teratomas we can find pyknotic nuclei that indicate apoptosis (Figure 44E and Figure 44F).

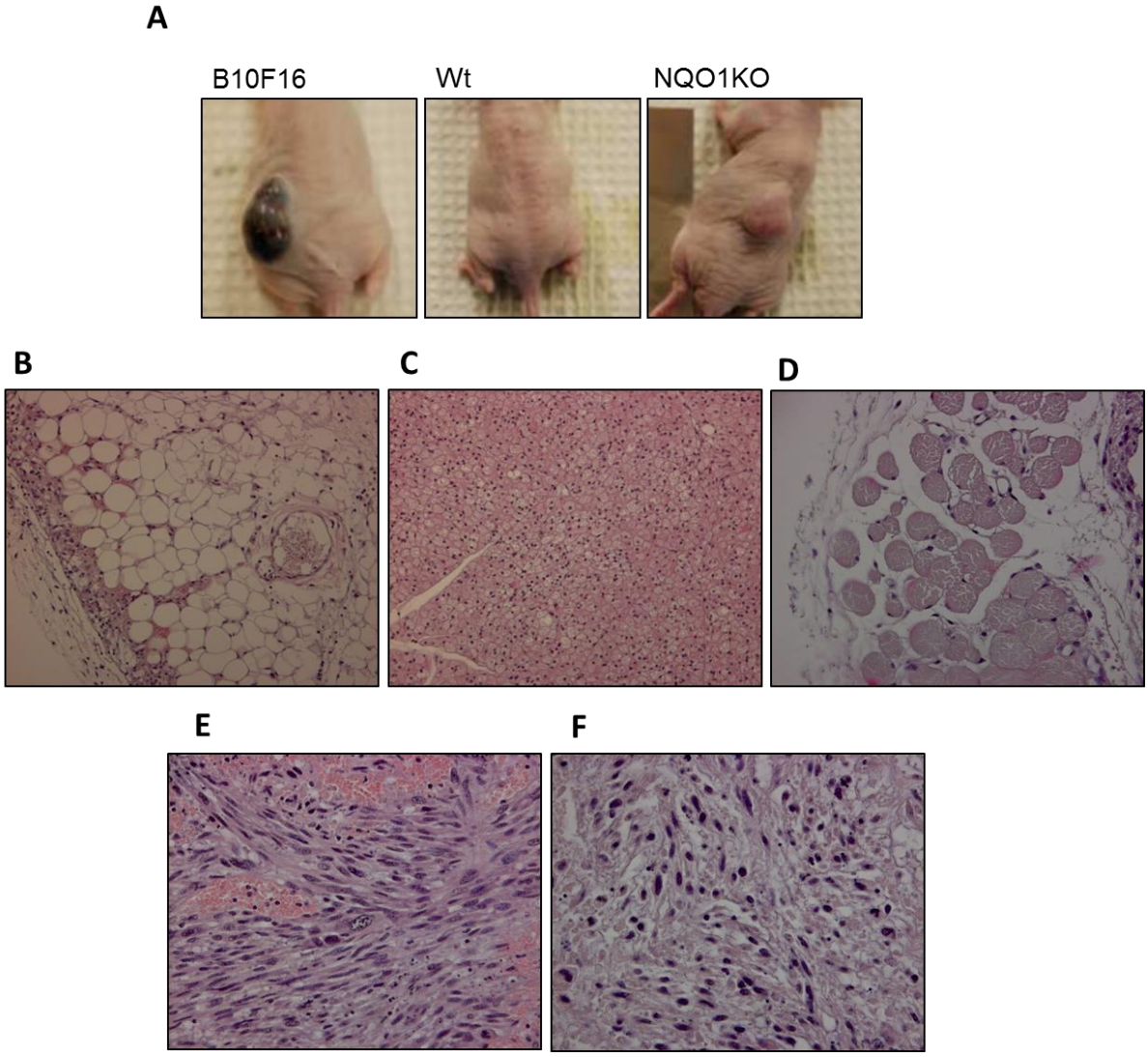

Figure 44: Tumor induction by cell injection into nude mice. A) Tumor generation: B10F16 cells were used as a positive control. Wild-type MEFs did not generate any tumors whereas NQO1KO MEFs produced teratoma-like tumors in 12 out of 13 injected mice. **B) White adipose tissue**. **C) Brown adipose tissue**. **D) Skeletal muscle tissue. E) and F) Fibroblastic-type cells**: Pyknotic nuclei in fibroblasts-like areas of teratomas.

Discussion

1. Effects of Nrf2 genetic deletion

1.1. Basal apoptosis is increased in MEFs bearing a genetic deletion of Nrf2 gene and in tissues from Nrf2KO mice

Nrf2 has been recently shown to control the expression of several antiapoptotic proteins, as Bcl-2 and Bcl-xL (Niture and Jaiswal 2012, Niture and Jaiswal 2013). Thus we hypothesized that decreased growth of Nrf2KO MEFs could be due to enhanced rates of basal apoptosis. We aimed to demonstrate that genetic deletion of Nrf2 actually increases apoptosis in the absence of pro-apoptotic stimuli. Previous work in the field have reported that the inhibition of Nrf2-dependent transcription by gene silencing is linked to the enhancement of etoposide and radiation-induced apoptosis in mouse and human hepatic tumor cells (Niture and Jaiswal 2012). In addition, Nrf2KO mice show higher levels of several pro-apoptotic factors in skeletal muscle from aged mice (Miller, Gounder et al. 2012, Narasimhan, Hong et al. 2014), although a putative increase of apoptosis of the skeletal muscle fibers was not tested directly in those previous studies. Of note, it has been recently reported that the expression of a constitutively active form of Nrf2 (caNrf2) delayed proliferation and induced apoptosis of hepatocytes in the regenerating liver of caNrf2-transgenic mice (Kohler, Kurinna et al. 2014), suggesting that a homeostatic balance of Nrf2 function is needed to protect mitotic tissues from apoptosis. In accordance, oncogenesis has been reported to increase in lung both by decreased expression of Nrf2 gene (Suzuki, Shibata et al. 2013) but also by unrestrained Nrf2 activation due to inactivating mutations in the *Keap1* gene (Ganan-Gomez, Wei et al. 2013). When we evaluate the levels of apoptosis, we found out that levels of basal apoptosis were increased in Nrf2KO MEFs and also in two mitotic tissues of Nrf2KO mice, lung and liver.

1.2. Mitochondrial apoptotic signaling is stimulated in Nrf2KO MEFs

The decisive event in the intrinsic apoptotic pathway is the permeabilization of the OMM that allows the release of several pro-apoptotic factors like cytochrome *c* and AIF to trigger the apoptotic cascade. Those factors are released through a Bcl-2 family-regulated structure, the mitochondrial apoptotic-induced channel (Kinnally and Antonsson 2007). The decrease of Bcl-2 levels in MEFs lacking Nrf2 we show here is in accordance with previous investigations demonstrating that the silencing of Nrf2 decreases Bcl-2 polypeptide in Hepa-1 and Hek-293 cells and, conversely, up-regulation of Nrf2 induces the expression of Bcl-2 and Bcl-xL (Niture and Jaiswal 2012, Niture and Jaiswal 2013). There is a direct relationship between Nrf2 and both Bcl-2 and Bcl-xL expression because these two antiapoptotic proteins are under transcriptional control by Nrf2 that binds to an ARE located in the promoter region of the corresponding genes (Niture and Jaiswal 2012, Niture

and Jaiswal 2013). Regarding Bax, it is unclear whether or not there exists a direct link between Nrf2 and Bax expression. In a cellular *in vitro* model, it was previously shown that experimental augmentation of Nrf2 levels prevented Bax increase induced by treatment with etoposide. However, Bax levels were not significantly altered in cells that had not been treated with the apoptosis inductor (Niture and Jaiswal 2013). This agrees with the lack of Bax changes in Nrf2KO MEFs we report here. Conversely, although Bax polypeptide was significantly increased in skeletal muscle from aged (> 24 months of age) Nrf2KO mice (Miller, Gounder et al. 2012, Narasimhan, Hong et al. 2014), it is not clear if this Bax increase is a direct effect of Nrf2 deficiency or it is an indirect effect related with aging (Miller, Gounder et al. 2012). Strikingly, Bim, another proapoptotic member of Bcl-2 family, is also positively regulated by Nrf2 as part of a protective response against hyperproliferative conditions that may prevail due to constitutive Nrf2 activation in liver from caNrf2 transgenic mice (Kohler, Kurinna et al. 2014).

The significant decrease of Bcl-2 levels and Bcl-2/Bax ratio observed in Nrf2KO MEFs is consistent with an enhancement of the mitochondrial pro-apoptotic pathway in these cells. Release of cytochrome *c* to the cytosol together with dATP and apoptotic protease-activating factor-1 (Apaf-1) association promotes assembly of the apoptosome which triggers a caspase cascade *via* activation of caspase-9 (Bratton and Salvesen 2010), whereas the release of apoptosis-inducing factor (AIF) to the cytosol and its translocation to the nucleus is sufficient to facilitate DNA fragmentation through a caspase-independent pathway (Susin, Lorenzo et al. 1999). To our knowledge, this is the first demonstration that genetic deletion of Nrf2 increases cytosolic cytochrome *c* and nuclear AIF levels in mitotic cells. Furthermore the increase of nuclear AIF observed in Nrf2KO MFEs agrees with the AIF increase in skeletal muscle from aged Nrf2KO mice (Miller, Gounder et al. 2012). In addition, we found that Apaf-1 levels were significantly increased in Nrf2KO MEFs, which supports that Nrf2 may participate in the regulation of Apaf-1 levels by decreasing its abundance in cytosol. Expression of the APAF1 gene is subjected to a very complex regulation including transcriptional regulation by E2F-1 and p53, epigenetic regulation by histone deacetylases and methylation of the APAF1 gene and suppression by several miRNAs (Wallace and Cotter 2009, Chen, Xu et al. 2014, Yong, Wang et al. 2014). How Nrf2 might interact with any of these systems known to regulate Apaf-1 levels remains for further investigation.

In order to evaluate the DNA damage with another method different from the ELISA (apoptotic index), we performed an immunocytochemical assay to quantify the γH2A.X histone. This is a way to quantify DNA double strand breaks (DSBs). It has been reported that γH2AX is required for the accumulation of many DNA damage response (DDR) proteins

at DSBs. Thus, γH2AX is believed to be the principal signaling protein involved in DDR, playing an important role in DNA repair (Yuan, Adamski et al. 2010). The increase in γH2AX signal intensity in the Nrf2KO MEFs is consistent with higher DNA fragmentation.

With respect to intracellular peroxide levels, we found that the small increase of ROS in Nrf2KO MEFs did not reach statistical significance when compared to wild-type MEFs. This is in contrast with another study carried out with type II alveolar cells obtained from Nrf2KO mice, in which the deletion of Nrf2 resulted in a dramatic increase of ROS levels (Reddy, Kleeberger et al. 2007). Type II alveolar cells are constantly exposed to an oxidant environment because of their roles in host defense, injury, and repair processes (Reddy, Kleeberger et al. 2007). It has been also established that this type of cells are involved in the tumorigenesis process of lung cancer, being susceptible to undergo K-Ras-induced carcinogenesis (Xu, Rock et al. 2012). Moreover, alterations of the Nrf2 status in lung, either related with decreased gene expression or with Nrf2 upregulation due to Keap1 mutations, is linked to human lung cancer susceptibility (Suzuki, Shibata et al. 2013). This suggest that the extent of ROS alterations caused by Nrf2 deletion might be cell-type dependent. In addition, other compensatory mechanisms could have arisen during the long-term culture of MEFs in order to compensate for the loss of Nrf2. The possibility exist that these compensatory changes could involve Nrf1 since both Nrf1 and Nrf2 are major regulators of cytoprotective gene expression (Leung, Kwong et al. 2003) and Nrf1 is also able to trigger the ARE-dependent response as well (Motohashi, O'Connor et al. 2002).

1.3. Activation of the mitochondrial caspase cascade is blunted in Nrf2KO MEFs

Release of mitochondrial proapoptotic factors leads to activation of a caspases cascade for the proteolytic degradation of many cellular substrates (Crawford and Wells 2011). Apoptosome-dependent activation of caspase-9 and the subsequent cleavage and activation of the executioner caspase-3 are key factors of mitochondrial (intrinsic) pathway apoptosis which takes part in cell removal after ionizing radiation exposure, DNA and mitochondrial damage or growth factor withdrawal (Boatright and Salvesen 2003, Bratton and Salvesen 2010). On the other hand, caspase-8 is the prototypic caspase that is activated through the plasma membrane-dependent extrinsic pathway, typical of immune-system tumor removal, immune system education and development (Kantari and Walczak 2011). Since Nrf2KO cells showed substantially increased levels of cytosolic cytochrome *c* and Apaf-1, it is reasonable to argue that activities of caspases-9 and -3 would be also upregulated. However, proteolytic activities of caspases-9 and -3 were not increased significantly in Nrf2KO MEFs in comparison with the control cells, despite the substantial decrease of Bcl-2/Bax ratio and

the elevation of cytosolic cytochrome *c* and Apaf-1 levels in the KOs. On the other hand, a significant increase of caspase-8 activity was found in Nrf2KO MEFs.

However, it has to be taken into account that these results were obtained by performing caspase enzymatic assays with fluorogenic peptide substrated. These kind of assays have been criticized in some instances because of their low specificity (Ehrnhoefer, Skotte et al. 2011). Indeed, substantial residual caspase-like activity can be obtained even in the presence of high amounts of inhibitory peptides, indicating that other proteases could also participate in the proteolytic cleavage of the fluorogenic peptides. As a consequence, we decided to study caspase activation by a western blot technique with specific antibodies recognizing both the activated cleaved caspases and the full-length uncleaved procaspases. This approach allowed us to evidence that procaspase-9 was significantly elevated and procaspase-8 decreased in Nrf2KO MEFs. A trend towards an increase of procaspase-3 was also observed. Because the components of the caspase activation system are constitutively expressed in the majority of cells and the activation of caspases is a post-translational process, substantial interest has been paid to the factors controlling proteolytic activation of procaspases. However, the mechanisms involved in transcriptional regulation of caspases gene expression are not fully understood (Kumar and Cakouros 2004). Interestingly, the increase of procaspases-9 levels in Nrf2KO MEFs is in agreement with the increase of Apaf-1 levels in the same cells because these proteins are transcriptionally controlled by the same factors, including E2F1 and p53 (Kumar and Cakouros 2004).

Cleaved activated caspases could be only revealed in these western blots after prolonged developing of the enhanced chemiluminiscence reaction, which suggests the presence of low levels of activated caspases in the cells. Anyway, the results obtained for cleaved caspases were in full agreement with the enzymatic assays of caspase activity, *i.e.* no statistically significant differences were observed between wild-type and Nrf2KO cells for caspase-9 (a trend to increase) and caspase-3, whereas a significant increase of cleaved caspase-8 was observed for the KOs.

Since an increase of caspase-8 activity (and activated cleaved caspase-8 fragments) was the most prominent difference between wild-type and Nrf2KO MEFs regarding the activation of the caspase cascade, we wanted to investigate if activation of this caspase was related with the increase of basal apoptosis in Nrf2KO MEFs. BH3-interacting domain death agonist (Bid) is a Bcl-2 family pro-apoptotic member known to undergo cleavage by caspase-8 to generate truncated Bid (tBid), a 15 KDa fragment which interacts with the OMM leading to OMM permeabilization and apoptosis (Luo, Budihardjo et al. 1998, Billen, Shamas-Din et al.

2008, Kantari and Walczak 2011). Also, tBid stimulates the caspase-independent apoptotic pathway (van Loo, Schotte et al. 2001). Cleavage of Bid by caspase-8 is the link between extrinsic apoptotic pathway and activation of the mitochondrial apoptosis in type II cells, whereas caspase-8 directly activates executioner caspase-3 in type I cells (Luo, Budihardjo et al. 1998, Walter, Schmich et al. 2008, Jost, Grabow et al. 2009, Kantari and Walczak 2011). MEFs are well characterized type II cells (Walter, Schmich et al. 2008). Thus, we tested the effect of Z-IETD-FMK, a non-toxic irreversible inhibitor of caspase-8, on DNA fragmentation. Our results indicated that the amount of cytosolic oligonucleosomal fragments was unchanged in cells treated with Z-IETD-FMK at concentrations ranging from 5 to 25 μM. Since full inhibition of caspase-8 in fibroblasts by Z-IETD-FMK has been reported at concentrations above 2μM (Schull, Gunther et al. 2015), our results support that activation of caspase-8 is not related with the elevation of basal apoptosis in Nrf2KO MEFs. On the other hand, caspase-8 plays a number of non-apoptotic roles in cells, such as promoting activation NF-κB signaling, regulating autophagy and altering endosomal trafficking, and enhancing cellular adhesion and migration (Stupack 2013). How these cellular functions may be altered in Nrf2KO cells, and the putative involvement of caspase-8 activation in these alterations remain for further investigation.

In sum, our results support that mitochondrial apoptotic signaling is blunted at the level of caspases cascade activation in cells lacking Nrf2. It is thus very likely that the increase of DNA fragmentation observed in Nrf2KO MEFs could be mediated by increased nuclear levels of AIF, as it has been demonstrated that AIF is able to induce chromatin condensation and fragmentation in a caspase-independent pathway (Susin, Lorenzo et al. 1999).

One possibility to explain the lack of caspase-3 activation would involve the participation of the Inhibitors of Apoptotic Proteases (IAPs). One of the main IAPs is the X-chromosome linked Inhibitor of Apoptotic Proteases (XIAP), known to behave as a potent inhibitor of caspase-9 and effector caspases (Jost, Grabow et al. 2009). , We found no change of XIAP levels among wild-type and Nrf2KO MEFs. However, when cells were treated with BV6, a Smac mimetic and XIAP inhibitor, wild-type and Nrf2KO MEFs behaved in a different way. The induction of cell death in Nrf2KO, but not in wild-type MEFs, when treated with the Smac mimetic can be due to the fact that cells lacking Nrf2 have a very high content of pro-apoptotic factors although the intrinsic caspase pathway is blunted, most likely because XIAP levels are enough to block the caspase cascade at the caspase-3 level. It is demonstrated that procaspase-9 possesses higher affinity for the apoptosome complex than the activated caspase-9, and can displace the processed caspase-9 from it. Thus, the apoptosome functions as a proteolytic-based 'molecular timer', in which the intracellular

concentration of procaspase-9 determines the duration of this timer. Autoprocessing of procaspase-9 activates the timer and the rate at which the processed caspase-9 dissociates from the complex and subsequently loses the capacity of activating caspase-3 (Malladi, Challa-Malladi et al. 2009). According to this report, higher levels of procaspase-9 prevents caspase-3 activation and this could be the situation in the Nrf2KO MEFs together with high levels of XIAP. In accordance, in the presence of a XIAP inhibitor such as BV6, it is possible to experimentally activate the apoptotic cascade in Nrf2KO MEFs.

1.4. MAPK and Akt pathways are altered in Nrf2KO MEFs

Mammalian MAPK pathways can be activated by a wide variety of different stimuli through diverse receptor families and are responsible of a number of functions including apoptosis (Kyriakis and Avruch 2012). It is well known that the Nrf2-Trx1 axis controls the function of stress-activated protein kinases since Trx1, one of the main transcriptional targets of Nrf2, binds and inhibits Apoptosis signal-regulating kinase 1 (Ask1), a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family involved in both the stress response and apoptosis that activate JNK and the p38 MAPK family (Piccirillo, Filomeni et al. 2009, Niso-Santano, Bravo-San Pedro et al. 2011). Interestingly, previous studies have documented that the levels of Ask1 are increased in skeletal muscle from old Nrf2KO mice, and this increase is exacerbated by exercise (Miller, Gounder et al. 2012, Narasimhan, Hong et al. 2014). However, how the lack of Nrf2 may affect the phosphorylation state of the main MAPK families, and if these putative changes in MAPK activation participate or not in apoptosis engagement in cells without Nrf2 function have not been addressed to date. Within this context, we have studied if the lack of Nrf2 altered phosphorylation state of the stress protein kinases p38 and JNK1. In addition, we also studied phosphorylation state of ERK1/2 since, besides being activated by mitogenic signals, this MAPK can be also activated by environmental stress such as oxidative stress, (Kyriakis and Avruch 2012). In addition, our study also included Akt, a protein involved in a pathway related to proliferation and survival, protecting cells against apoptosis (Altomare and Khaled 2012). Genetic deletion of Nrf2 produced significant phosphorylation of ERK1/2 and p38, but phosphorylation states of JNK1 remained unaltered and Akt presented a trend to decrease. These changes could be due to the oxidative stress caused by the deletion of Nrf2 and the long-term culture of the cells.

Many apoptotic responses have been shown to be dependent on p38 activation (Nebreda and Porras 2000, Porras, Zuluaga et al. 2004, Kralova, Dvorak et al. 2008). Fibroblasts lacking p38 are more resistant to apoptosis induced by different stimuli (Porras, Zuluaga et al. 2004). On the other hand, ERK1/2 signaling is also an important regulator of apoptosis,

and influences the expression and/or activity of many members of the Bcl-2 protein family, in many cases to promote survival (Balmanno and Cook 2009). However, induction of apoptosis may be also mediated by ERK1/2- in other experimental models (Hsieh, Chien et al. 2014, Park, Kim et al. 2015). Our results with ERK1/2 phosphorylation are consistent with previous observations carried out in immature dendritic cells, in which enhanced ERK1/2 phosphorylation state was found when Nrf2 was deleted (Aw Yeang, Hamdam et al. 2012). However, the fact that DNA fragmentation was not affected by p38 and ERK1/2 inhibitors may be indicative that the upregulation of the phosphorylation degree of these kinases might not be decisive in determining cell fate towards survival or death in our experimental model.

1.5. p38 modulates Bcl-2 levels in wild-type but not in Nrf2KO MEFs

Activation of p38 sensitizes cells to apoptosis *via* both up-regulation of pro-apoptotic proteins and down-regulation of survival pathways (Porras, Zuluaga et al. 2004). Interestingly, p38 can target the function of anti-apoptotic Bcl-2 by two independent although related mechanisms. Firstly, p38 is a negative transcriptional regulator of Bcl-2 abundance. *BCL2* transcripts and Bcl-2 protein levels are significantly increased in mouse embryonic stem cells under conditions of p38 activity repression caused by either genetic deletion of the p38α gene or by pharmacological inhibition with SB203580 or PD169316 (Trouillas, Saucourt et al. 2008). Secondly, Bcl-2 protein itself is a target of p38-mediated phosphorylation in Ser^{87} and Thr^{56} residues, and phosphorylation of these residues is associated with a decrease in the anti-apoptotic potential of Bcl-2 (De Chiara, Marcocci et al. 2006). Thus, we wanted to investigate if besides a decrease of Bcl-2 directly dependent on Nrf2 deletion, increased p38 activation observed in Nrf2KO MEFs might contribute additionally to stimulate apoptosis by further diminishing Bcl-2 levels and increasing its phosphorylation degree. If this possibility was true, then p38 inhibition would allow to restore, at least partially, Bcl-2 levels in Nrf2KO MEFs. Thus, we measured levels of total and of Ser^{87}–phosphorylated Bcl-2 in wild-type and Nrf2KO MEFs, both in the absence and in the presence of the two p38 inhibitors. The increased Bcl-2 levels and decreased phosphorylation degree in wild-type MEFs fully agrees with the dual role of p38 in the regulation of Bcl-2 function (De Chiara, Marcocci et al. 2006, Trouillas, Saucourt et al. 2008). However, the fact that Bcl-2 levels remained equally low in these cells in Nrf2KO MEFs in the absence or the presence of p38 inhibitors supports that a functional Nrf2 pathway is required for the negative transcriptional regulation of Bcl-2 by p38. With respect to Bcl-2 phosphorylation in Ser^{87}, although it actually increased in Nrf2KO MEFs in comparison with wild-type cells (which agrees with greater p38 activation in the Nrf2KOs), it also remained

elevated in the presence of SB203580 or PD169316, indicating that additional kinases besides p38 may be involved in Bcl-2 phosphorylation in Nrf2KO MEFs.

Interestingly, previous studies have demonstrated that phosphorylation of Nrf2 by p38 produces different outcomes depending on the model of study. In some cases, p38 has been reported to promote the dissociation of Nrf2 from the Nrf2–Keap1 complex, either directly by phosphorylating Nrf2 or indirectly through intermediary kinases, resulting in greater Nrf2 accumulation in the nucleus and the activation of some antioxidant target genes (Martin, Rojo et al. 2004). However, other studies have described a different response with no role for p38 on Nrf2 activation (Li, Wu et al. 2011) or even the inhibition of Nrf2 transcriptional activity by p38 (Shen, Hebbar et al. 2004, Song, Li et al. 2014). In MEFs, it has been previously reported that p38 negatively regulates heme oxigenase-I (HO-I) expression through inhibition of Nrf2 (Naidu, Vijayan et al. 2009). This is consistent with a negative role for p38 in regulating Nrf2 function in this cell type and agrees with our observation that Bcl-2 levels were increased in wild-type MEFs when p38 activity was inhibited with either SB203580 or PD169316. We also studied how the Nrf2 status affected the levels of Trx1, another well-established target of Nrf2 (Im, Lee et al. 2012) and how p38 inhibition affected Trx1 levels in wild-type and Nrf2KO MEFs. If p38 is a negative effector in regulating Nrf2 function in MEFs and this MAPK controls Bcl-2 expression specifically through its action on Nrf2, then the two p38 inhibitors should also increase Trx1 levels in wild-type but not in Nrf2KO MEFs. The decrease of Trx1 levels in Nrf2KO MEFs is consistent with the activation of p38 through the Nrf2-Trx1-Ask1 axis (Piccirillo, Filomeni et al. 2009, Niso-Santano, Bravo-San Pedro et al. 2011) in which Trx1 inhibits Ask1 activation, and hence p38 phosphorylation. As observed for Bcl-2, a substantial elevation of Trx1 levels by p38 inhibitors was observed in wild-type but not in Nrf2KO MEFs. In accordance with our observations, a previous study has demonstrated that inhibition of p38 induced binding of nuclear proteins to a Nrf2 target sequence of the HO-1 promoter, but it did not affect HO-1 promoter activity and protein expression in Nrf2KO MEFs (Naidu, Vijayan et al. 2009).

In summary we demonstrate that p38 is clearly a negative effector of Bcl-2 function in wild-type MEFs through the inhibition of Nrf2 transcriptional activity and the phosphorylation of Bcl-2. However, this anti-apoptotic protein becomes independent on p38 activity in Nrf2KO MEFs.

1.6. Monoamine oxidase participates in apoptosis engagement in Nrf2KO MEFs

MAO activity has been related with mitochondrial permeabilization, which can be abolished by inhibitors of both MAO-A and B in rat liver mitochondria (Marcocci, De Marchi et al. 2002). Also Nrf2 has been described to downregulate MAO B mRNA in a transcriptomic study (Reddy, Kleeberger et al. 2007). Taken into consideration these premises, we hypothesized that MAO-B could be involved, at least in part, in the increased mitochondrial permeabilization observed in Nrf2KO MEFs.

MAO-A and MAO-B are differentially regulated by several transcription factors and signaling pathways (Chen 2004, Shih and Chen 2004). There is substantial evidence that the expression of MAO-A is induced through p38-dependent pathways in several cell types and during apoptosis (De Zutter and Davis 2001, Ou, Chen et al. 2006), although phosphorylation in a p38-consensus motif has been also reported to inhibit MAO-A activity in several neuronal cell lines (Cao, Rui et al. 2009).

To investigate is any of the MAO isoforms was involved in the stimulation of basal apoptosis in Nrf2KO MEFs, we carried out a pharmacological approach with inhibitors known to affect either MAO-A or MAO-B. The results we obtained with chlorgyline (MAO-A inhibitor) and deprenyl (MAO-B inhibitor) on DNA fragmentation in wild-type MEFs suggest that MAO-A might play a pro-apoptotic role in MEFs but is not related with the increase of basal apoptosis in cells lacking Nrf2. Cao et al. (2009) demonstrated that over-activation of p38 could lead to an inhibitory phosphorylation of MAO-A. It could be postulated that a similar mechanism could be operative in Nrf2KO MEFs, which exhibit increased phosphorylation of p38. Nevertheless, the lack of effect of p38 inhibitors on DNA fragmentation in Nrf2KO MEFs makes it unlikely that MAO-A plays any prominent role in determining basal apoptotic rates in these cells. The status of MAO-A in Nrf2KO MEFs remain for further investigation.

With regards to the MAO-B inhibitor, it was found that deprenyl not only decreased DNA fragmentation in wild-type, but also in Nrf2KO MEFs. A proapoptotic role for MAO-B in Nrf2KO MEFs agrees with the previous demonstration that Nrf2 suppresses MAO-B expression (Reddy, Kleeberger et al. 2008). However, it is important to consider that different mechanisms have been reported to explain deprenyl protection of cultured cells from apoptosis induced by various stimuli. In some cases, induction of anti-apoptotic proteins synthesis has been proposed to explain the protective effect of deprenyl when used at very low concentrations (10^{-9} to 10^{-11}M) which are unable to inhibit MAO-B (Tatton, Ju et al. 1994, Malorni, Giammarioli et al. 1998, Suuronen, Kolehmainen et al. 2000). However,

this effect is lost at the concentrations (10^{-5}) where we found maximal action against apoptosis in Nrf2KO MEFs. In addition, at concentrations ranging from 50 to 100μM, deprenyl has been demonstrated also to prevent MPP^+-induced oxidative damage in PC12 cells by the upregulation of Nrf2-mediated NQO1 expression through the activation of PI3K–Akt and ERK (Xiao, Lv et al. 2011). Obviously, this pathway is not involved in the decrease of basal apoptosis by deprenyl we have observed here because Nrf2KO MEFs completely lack this transcription factor and thus, this pathway is necessarily blunted. Moreover, deprenyl was completely ineffective at 10μM in activating Nrf2 in PC12 cells, but concentrations ranging from 50 to 100μM were required to activate this protective pathway. We found these concentrations of deprenyl toxic for the MEFs and a significant decrease of DNA fragmentation in Nrf2KO MEFs was already obtained at 1-10μM. Finally, at a much higher concentration (500μM) deprenyl has been reported to affect mitochondrial physiology in a more generalized way because of the inhibition of mitochondrial membrane permeability transition though a mechanism that does not involve MAO-B (De Marchi, Pietrangeli et al. 2003). However, these assays were not carried out in cellular systems but with isolated liver mitochondria. Thus, it is possible that this mechanisms does not have physiological relevance due to the very high concentrations of inhibitor used.

Since significant inhibition of DNA fragmentation in Nrf2KO MEFs was already achieved at 1μM deprenyl, our results support that MAO-B activity could mediate the pro-apoptotic effects of Nrf2 genetic deletion. The identification of a MAO-B inhibitor as an effective pharmacological agent able to abate the increase of DNA fragmentation in Nrf2KO MEFs together with the observation that Nrf2 downregulates MAO-B transcripts (Reddy, Kleeberger et al. 2007) prompted us to investigate if MAO-B protein levels are indeed altered in these cells. In accordance with observations, Nrf2KO MEFs showed higher levels of MAO-B, supporting that this enzyme could be involved in damaging mitochondria in Nrf2KO cells.

1.7. Mitochondria show ultrastructural alterations in Nrf2KO MEFs

PGC1α is a mitochondrial function activator. Alterations in PGC1α levels have been associated to several pathologies like Alzheimer's or Parkinson's disease and even type II diabetes type or obesity (Handschin and Spiegelman 2006). Some of them are strongly linked to the mitochondrial dysfunction like neurodegenerative diseases or aging (reviewed by (Austin and St-Pierre 2012)). It is well established that overexpression of PGC1α is directly related with an increase in mitochondria volume and cristae density (Rohas, St-Pierre et al. 2007). Our observation that Nrf2KO MEFs had lower levels of PGC1α is consistent with a decrease of PGC1α levels previously found in skeletal muscle from aged

Nrf2KO mice in comparison with age-matched controls (Narasimhan, Hong et al. 2014). PGC1α activity is also regulated by acetylation. It has been demonstrated that deacetylase sirtuin1 (Sirt1) directly interact with PGC1α and regulates acetylation levels of PGC1α both *in vivo* and *in vitro*, inhibiting its transcriptional activity (Nemoto, Fergusson et al. 2005). Previous results in our group showed how Sirt1 was downregulated in immortalized Nrf2KO MEFs (Jodar, Mercken et al. 2011). Thus, although lower levels of PGC1α can be found in Nrf2KO MEFs, its putative higher acetylation degree could result in a more active protein. The study of PGC1α acetylation degree in MEFs lacking Nrf2 remains for future research.

In our ultrastructural analysis focused on mitochondria we have found that Nrf2KO MEFs mitochondrial area was smaller if compared with their wild-type counterparts. However, at a cellular level mitochondria were more abundant and there was a higher surface of cytoplasm occupied by these organelles. This suggests that the higher number of mitochondria in Nrf2KO MEFs could be a consequence of alterations in the fusion/fission process. In our hands, the fusion and fission marker proteins that we have measured evidenced a decrease in fusion and no differences in fission markers. This unbalance in the mitochondrial dynamic markers could lead to a less fused mitochondria situation that could explain the higher numbers of mitochondrial profiles in Nrf2KO MEFs when compared to their wild-type counterparts. The inhibition of mitochondrial fusion and subsequent mitochondrial fragmentation seem to potentiate mitophagy and cell death. However, the opposite state mediates the formation of a highly connected mitochondrial network and is thus thought to buffer the potentially deleterious effects of stresses (Friedman and Nunnari 2014).

Osmotic swelling is one of the fundamental characteristics exhibited by mitochondria in pathological situations, which activates downstream cascades that culminates in apoptosis. In fact, a mechanism involving aquaporins have been suggested (Lee and Thevenod 2006). The osmotic swelling is consistent with an enhanced-apoptotic situation and also this mechanism could explain the higher surface of cytoplasm occupied by mitochondria in the Nrf2KO in comparison to the wild-type MEFs. Lower levels of the fusion protein Mfn2 are also compatible with a study that describe that silencing of Mfn1/2 causes the generation of shorter mitochondria and the increase of apoptotic levels (Sugioka, Shimizu et al. 2004). Thus, Mfn2 decrease is consistent with the reduction of mitochondrial size in Nrf2KO MEFs and also with the increase of apoptosis in these cells.

2. NQO1 genetic deletion

2.1. NQO1KO MEFS display longer life span. A role for Nrf2?

We decided to assess the ability to grow in long-term cultures of MEFs, both of wild-type and NQO1KO genotypes. Previous results obtained in our group had shown that wild-type MEFs grew steadily until passage 62 but presented a proliferation limit at 64 passages. However, for MEFs lacking NQO1 the proliferation limit was reached at around 80 passages. The experimental procedures described in this chapter were carried out with immortalized NQO1KO MEFs obtained from passages between 25 and 35. Our data have evidenced that NQO1KO MEFs are more resistant than the wild-type MEFs against environmental stresses that characterize a long-term culture. Their longer life span together with the higher proliferation rates of these cells are consistent with a role for the lack of NQO1 in tumor progression. Nrf2 signaling has been suggested to lighten the effects of oxidative stress (including some aging-associated diseases like neurodegeneration, chronic inflammation, and cancer both in rodents and humans). In a cellular model of human fibroblasts it has been shown that Nrf2 has a declined function in senescence and also that Nrf2 silencing lead to premature senescence. Moreover, treatment with a Nrf2 inducer resulted in the enhanced survival of cells following oxidative stress, whereas continuous treatment lead to life span extension of human fibroblasts (Kapeta, Chondrogianni et al. 2010). Another study carried out with human fibroblasts has also linked the increase of Nrf2 due to a rapamycin treatment with an extended life span (Lerner, Bitto et al. 2013). Moreover, activation of Nrf2 signaling has a prolongevity effect in *Drosophila megalonaster* males (Sykiotis and Bohmann 2008, Sykiotis and Bohmann 2010). Interestingly, Nrf2-dependent signaling has been also identified as a major determinant of cellular stress resistance in a model of mice with extended longevity (Leiser and Miller 2010). Therefore, higher levels of Nrf2 might constitute a growth advantage that results in an extended life span in the long-term culture. In addition, it has been reported that mice null for another detoxifying enzyme, the transferase mGsta4, present chronically elevated Nrf2 levels that seem to have an effect on extending the life span of these mice as a compensatory response to the lack of mGsta4 (Singh, Niemczyk et al. 2010). We hypothesized that the latter situation could be similar to the events taking place in NQO1KO MEFs as well. In accordance to this hypothesis, we observed a higher nuclear translocation of Nrf2 in MEFs and also in liver of NQO1KO mice. The possibility exists that this increase of nuclear Nrf2 could have been developed as a hormetic response to compensate the lack on the antioxidant enzyme NQO1.

The Nrf2 activation in cells lacking NQO1 could be due to different stimuli and complex interactions. The slightly increased levels of p62 in the NQO1KO MEFs might play a role in

Nrf2 sustained activation. It has been described that p62 takes part in the non-canonical Nrf2 activation pathway. Actually, this signaling cascade (p62/Nrf2/NQO1) has been suggested as a way to assure mammalian longevity through mitochondrial integrity (Kwon, Han et al. 2012), being the latter consistent with an alteration in size and mitochondrial dynamics in the NQO1KO MEFs. Increased p62 has been also linked with increased tumorigenesis and with an increase of the tumor size (Moscat and Diaz-Meco 2009). In fact, an enhancement of the p62/Nrf2 axis correlates with bad prognosis and tumor progression in human gliomas (Zhao, Xu et al. 2015).

2.2. NQO1 deletion enhances the apoptosis process

As previously indicated, NQO1KO cells exhibit higher doubling rates than their wild-type counterparts. This might be indicative of a decrease in apoptosis and/or an increase in proliferation. Surprisingly, in our experimental model we have observed higher levels of basal apoptosis in NQO1KO MEFs. However, concomitance of apoptosis and excessive growth has been also observed in certain types of tumors which present an uncontrolled growth that led to high levels of apoptosis as well. In fact in liver, there is evidence that apoptosis favors the carcinogenic process and that inhibition of the apoptotic process can help to prevent carcinogenesis (Hikita, Kodama et al. 2012). In another *in vitro* model using mouse keratinocytes, it has been reported that the genetic deletion of NQO1 enhances TNF-induced although not basal apoptosis (Ahn, Sethi et al. 2006). Similarly, some apoptotic markers such as cytochrome *c* or AIF were increased in livers of NQO1KO mice upon challenging with acetaminophen (Hwang, Kim et al. 2014).

As indicated, one of the most important event in the apoptotic process is the permeabilization of the OMM that allows the release of several pro-apoptotic factors like cytochrome *c* and AIF to trigger the apoptotic cascade. The release of these factors takes place through channels formed by a Bcl-2 family structure (Kinnally and Antonsson 2007). In this work we have assessed the levels of three Bcl-2 family proteins and major regulators of the mitochondrial apoptotic pathway: Bcl-2, an anti-apoptotic protein; Bax, a pro-apoptotic factor, and Bid, a BH3-only proapoptotic polypeptide. All of them interact with the OMM and thus, regulate the propagation of the apoptotic signal by disruption of its integrity (Chipuk, Moldoveanu et al. 2010). In our study we found that Bax levels did not change by deletion of NQO1. However, we observed an increase in Bcl-2 levels in NQO1KO. When we calculated the ratio Bcl-2/Bax, an indicator of the enhancement of the intrinsic pathway, the same tendency to increase was maintained in immortalized NQO1KO MEFs, indicating certain anti-apoptotic effect. It has been reported that Bcl-2 possesses an ARE sequence (Niture and Jaiswal 2012). Consistently, we have also found that Nrf2 nuclear translocation

is increased both in MEFs and liver from NQO1KO animals. Therefore, the induction of Nrf2 can subsequently positively regulate Bcl-2. In NQO1/NQO2 double KO mice, an increase of Bcl-2 protein levels have been also shown (Shen, Barrios et al. 2010). However, lower levels of Bcl-2 have also been reported in NQO1KO keratinocytes (Ahn, Sethi et al. 2006). This might indicate that elevated levels of Bcl-2 are not a direct effect of NQO1 deletion, but a consequence of the compensatory increase of Nrf2.

However, an increase in the Bcl-2/Bax ratio is not consistent with the cytosolic release of cytochrome *c* and the increase of the nuclear AIF in NQO1KO MEFs. For this reason we also analyze the levels of Bid, another pro-apoptotic protein of the Bcl-2 family. It is known that full-length Bid undergoes an activating cleavage by caspase-8 that generates a 15 kDa fragment, tBid. The cleavage of Bid also leads the cell to OMM permeabilization and apoptosis (Luo, Budihardjo et al. 1998, Billen, Shamas-Din et al. 2008, Kantari and Walczak 2011) and facilitates translocation of AIF from the mitochondrial to the nucleus (van Gurp, Festjens et al. 2003). In our hands NQO1KO MEFs exhibited higher levels of full-length Bid. However, despite the enhancement of caspase-8 activation, no more tBid was generated in MEFs lacking NQO1. It has been described that, even though the full activity is acquired after Bid cleavage, full-length Bid also contains some pro-apoptotic function (Billen, Shamas-Din et al. 2008). The augmentation of full-length Bid levels could help to OMM permeabilization, as it is well-established that the pro- and anti-apoptotic Bcl-2 family proteins interact between each other to regulate the apoptotic response (Youle and Strasser 2008). The study of additional Bcl-2 family proteins might help to further understand the scenery. This remains for further investigation.

Data obtained with JC-1 probe support the hypothesis of an altered mitochondria polarization. JC-1 does not accumulate in mitochondria with depolarized $\Delta\psi$ but remains in the cytoplasm as monomers. A decrease in the mitochondrial potential ($\Delta\psi$) might indicates an altered mitochondrial function which may be due to apoptosis (Cossarizza, Baccarani-Contri et al. 1993). This decrease is consistent with the permeabilization of the OMM that leaks certain soluble IMS proteins, such as cytochrome *c*, into the cytoplasm. Similarly, decreased $\Delta\psi$ (with an increased cellular oxidant level) was also observed in NQO1-knockdown HCT116 cells (colon carcinoma cell line). Furthermore, ectopic expression of NQO1 completely restored $\Delta\psi$ as well as oxidant concentration in p62- (part of the non-canonical activation of Nrf2) or Nrf2-knockeddown HCT116 cells. This suggests a role for NQO1 in stabilizing mitochondrial integrity as a downstream effector of p62-induced basal Nrf2 activation (Kwon, Han et al. 2012).

Our data of the Bcl-2/Bax ratio and Bid, together with the results obtained with JC-1 probe and with the DNA fragmentation assay, suggest a higher OMM permeabilization. However, it is the release of pro-apoptotic factors after a permeabilization process of mitochondrial membranes the key event of mitochondrial apoptosis. Taken this together, we aimed to assess the permeabilization state of mitochondrial membranes in NQO1KO MEFs in comparison to their wild-type counterparts. We determined the levels of AIF in the nucleus after cell fractionation and observed that the levels of nuclear AIF increased significantly in NQO1KO when compared to wild-type MEFs. We also found that NQO1KO MEFs exhibited a significant increase in cytochrome *c* released to the cytosol. It is well-known that the release of AIF to the cytosol and its translocation to the nucleus is enough to facilitate DNA fragmentation through a caspase-independent pathway (Susin, Lorenzo et al. 1999). Consistent with our results, in a mouse model challenged with acetaminophen, NQO1KO mice showed increased levels of cytochrome *c* and AIF in liver. (Hwang, Kim et al. 2014).

Phosphorylation of histone variant H2AX at serine 139, named γH2AX, is widely used as a sensitive marker for DNA DSBs. However, H2A.X phosphorylation does not occur directly after DNA undergoes damage but it is mediated by the DNA damage response (DDR) (Sharma, Singh et al. 2012). DDR is vital for genomic stability. Defects in this system have been described as one of the most important hallmarks in cancer, and its disruption is observed in many of them (Pearl, Schierz et al. 2015). It is described γH2AX is required for the accumulation of many DDR proteins at DSBs. Thus, γH2AX is believed to be the principal signaling protein involved in DDR and to play an important role in DNA repair (Yuan, Adamski et al. 2010). A previous study carried out in HeLa cells showed that phosphorylation of H2A.X increases H2A.X stability preventing ubiquitylation and subsequent degradation. This augmented stabilization enhances H2A.X ability to block cell transformation (Zhu, Zykova et al. 2011). In order to evaluate the DNA damage with another method besides the ELISA DNA fragmentation assay, we performed an immunocytochemical assay to quantify the γH2A.X histone as a way to evidence DNA DSBs. Of note, we found no change among NQO1KO and wild-type MEFs. This lack of consistency of the H2A.X phosphorylation data and the DNA fragmentation could be due to a defect in the DDR of these cells. The lack of phosphorylation could be, a least partly, responsible of the NQO1KO MEFs phenotype, since the phosphorylation of H2A.X after the DNA damage blocks cell transformation.

With the purpose of elucidate the apoptotic pathways involved in DNA fragmentation of NQO1KO MEFs, we next analyzed how NQO1 deletion affected different caspase activities. Mitochondrial pathway was enhanced with activation of caspase-9 and -3, although cells

might also undergo caspase-independent apoptosis through AIF. However, despite the high activation of caspase-8, the extrinsic pathway initiator, the connection between extrinsic and intrinsic pathways is apparently blocked in NQO1KO MEFs. Despite the increase in caspase-8 activity there was not an increase in tBid. The cleavage of Bid by caspase-8 generating tBid is the link between extrinsic apoptotic pathway and activation of the mitochondrial apoptosis (Luo, Budihardjo et al. 1998, Kantari and Walczak 2011). MEFs are type II cells (Walter, Schmich et al. 2008) and thus, cleaved caspase-8 does not directly activate caspase-3. However, it is known that caspase-8 plays several cellular roles in vertebrates, not only mediating extrinsic apoptosis, but also regulating endosomal trafficking, autophagy and enhancing cell migration and adhesion (Stupack 2013). This can explain why normal or high levels of this caspase are found in several tumors (Barbero, Barila et al. 2008). The phosphorylation of a Tyrosine in the position 380 seems to be a key event in the diversion towards cell migration (Barbero, Barila et al. 2008). High levels of active caspase-8 correlate with the high healing capacity of NQO1KO cells. In addition, Nrf2 seems to have a role also in cell migration. Upon Nrf2 blockage, esophageal squamous cell carcinoma cells exhibit a repressed migration and invasion (Shen, Yang et al. 2014). The silencing of Nrf2 in human glioma cells U251 also led to a less invasive phenotype of the cells (Pan, Wang et al. 2013). Therefore, the higher Nrf2 nuclear translocation seems to promote cell migration. However, the opposite has been also shown in different oncogenic context in several tumor cells, including HepG2, A549, and SW480 cells, in which the loss of Nrf2 can also favor motility (Rachakonda, Sekhar et al. 2010).

2.3. Mitochondrial alterations of NQO1KO MEFs

NQO1KO MEFs presented smaller mitochondria if compared to the wild-type counterparts. However, these organelles were more abundant and occupied a higher surface proportion of cytoplasm. Alterations in the fusion/fission process might explain the mitochondrial structural differences between both genotypes. We have observed a decrease in two fusion proteins, Opa1 and Mfn2, but no differences in fission protein markers. It is reasonable to argue that this unbalance could cause less fused mitochondria. This is consistent with a study in which NQO1 was suggested to be needed for maintaining mitochondrial integrity. In that work, p62KO mice exhibited structural abnormalities of heart mitochondria, such as the appearance of electrodense matter or alignment disturbance (Kwon, Han et al. 2012). Mitochondrial fusion seems to be beneficial for the cell, buffering biochemical differences and having also implications in the ATP production (Hoitzing, Johnston et al. 2015).

Osmotic swelling of these organelles is one of the main features of apoptotic mitochondria (Lee and Thevenod 2006). The possibility exists that this process could play a role in

generating a higher number of mitochondria in the NQO1KO MEFs. Silencing of Mfn1 and Mfn2 leads to the shortening of mitochondria and the silencing process has also been linked to the apoptosis increase (Sugioka, Shimizu et al. 2004). The relation between the silencing of Mfn1 and Mfn2 proteins and the diminished mitochondrial area is consistent with our results in NQO1KO MEFs, and also with the existence of higher DNA fragmentation levels. In addition, the silencing of Opa1 by siRNA also leads to the fragmentation of the mitochondrial network and to cytochrome *c* release and apoptosis in HeLa cells (Olichon, Baricault et al. 2003). Besides Mfn2, Opa1 was also downregulated in NQO1KO MEFs, which could contribute to the alteration of the mitochondrial network in NQO1KO MEFs, resulting in less fused mitochondria and more apoptosis.

2.4. MAPK y Akt signaling pathways are altered in NQO1KO MEFs

Higher levels of apoptosis and proliferation rates might indicate the existence of an alternative mechanism by which the lack of NQO1 is compensated. Deregulation of mitogenic pathways could surpass this increase of apoptotic signaling resulting in the overall stimulation of growth rate and extended long-term survival of MEFs lacking NQO1. In the chemoprevention and carcinogenesis field, Nrf2 is a target for pharmacological inhibition in cancer and efficient Nrf2 inhibition has been shown to favor cell growth arrest and to sensitize cancer cells to apoptosis. Conversely Nrf2 stable upregulation also protects cancer cells from induced toxicity (Wang, Sun et al. 2008). This means that the upregulation of the Nrf2 pathway is a hormetic response elicited in order to compensate the lack of NQO1 which, in turn, leads to higher resistance against external insults. Importantly, the effects of Nrf2 on gene expression and metabolic activities are under the activation of PI3K–Akt signaling pathway. Akt is a protein involved in a pathway related to proliferation and survival (Altomare and Khaled 2012). The expression of Nrf2 in proliferating cells directs the enhancement of anabolic metabolism, maintains redox homeostasis and further promotes the activation of PI3K–Akt signaling, suggesting the presence of a positive feedback loop between Nrf2 and the PI3K–Akt pathway in proliferating cells (Mitsuishi, Motohashi et al. 2012). In the case of NQO1KO cells, we have observed higher levels of phospho-Akt and Nrf2 nuclear translocation. The increase of these two proteins might confer growth advantage to these cells. This is consistent with the extended long-term culture lifespan in MEFs lacking NQO1 as well as a higher proliferation rate.

Related to this increase in phospho-Akt is the increase of the mammalian monosaccharide transporters or Glucose Transport protein 1 (Glut1). Glut1 is ubiquitously expressed but seems to be especially upregulated in glycolytically active cells (Cura and Carruthers 2012). Compared to normal cells, cancer cells typically exhibit drastically increased glucose uptake

and glycolytic rates (Lu, Tan et al. 2015). Akt promotes cell proliferation by regulating different pathways, being one of them the regulation of Glut1 transcription and activity (Barthel, Okino et al. 1999, Wieman, Wofford et al. 2007). Tumor cells enhance glucose uptake *via* induction of Glut1 and coordinate the increased entry of glucose with increased glycolysis. Some studies have shown that the level of Glut1 expression correlates with specific tumor characteristics. Comparatively higher levels of expression are seen in cancers of higher grade and proliferative index and in cancers of lower degree of differentiation. This is because the more aggressive tumors are likely to have a greater demand for metabolic energy and thus for glucose. Accordingly, the expression level of Glut1 also correlates with a poor diagnosis cancer patients (reviewed by (Ganapathy, Thangaraju et al. 2009)).

Mammalian MAPK pathways can be activated by a wide variety of different stimuli through diverse receptor families and are responsible of a number of functions including apoptosis (Kyriakis and Avruch 2012). It is known that active Akt phosphorylates apoptosis signaling kinase 1 (Ask1) at Serine 83, which attenuates Ask1 activity and promotes cell survival. Ask1 transduces stress signals to the pro-apoptotic JNK and p38 MAP kinase cascades (Kim, Khursigara et al. 2001). These regulation pathway is consistent with the results we have found in NQO1KO MEFs. These cells exhibit higher levels of active Akt and decreased activated p38 and JNK1. ERK1/2 is activated by environmental stress such as oxidative stress and inflammatory cytokines rather than mitogens (Kyriakis and Avruch 2012), but in the case of NQO1KO MEFs we found no changes in the phosphorylation of ERK1/2. In addition to the inhibition of Ask1 by Akt-mediated phosphorylation, a separate mechanisms has been also described which is involved in this regulation. Trx1 is a well-known target of Nrf2 because of the presence of an ARE in the promoter region of the TRX1 gene (Im, Lee et al. 2012). It is well known that the Nrf2-Trx1 axis controls the function of stress-activated protein kinases since Trx1 binds to and inhibits Ask1. Thus, we also studied how the NQO1 status affected the levels of Trx1 in MEFs. Interestingly, Trx1 was increased in NQO1KO compared with wild-type MEFs, as we also observed for Bcl-2, another Nrf2 target. Higher levels of Nrf2 and Trx1 are consistent with the decrease of p38 phosphorylation in NQO1KO MEFs through activation of the Nrf2-Trx1-Ask1 axis (Piccirillo, Filomeni et al. 2009, Niso-Santano, Bravo-San Pedro et al. 2011). The activation of this antioxidant axis as a compensatory response is also consistent with the lower levels in intracellular peroxides observed in NQO1KO MEFs.

2.5. NQO1KO MEFs can induce tumors in nude mice

Another factor contributing to the phenotype of NQO1KO MEFs can be their low levels of activated (phosphorylated) p38. In a study with keratinocytes it was shown that NQO1 was needed for the TNF-induced JNK1 and p38 activation response (Ahn, Sethi et al. 2006). Dolado and colleagues characterized p38 as a ROS sensor and also highlighted its importance in the prevention of tumorigenesis in oncogene-transfected MEFs. These authors evidenced that MEFs lacking p38 were more prone to transformation by H-Ras (Dolado, Swat et al. 2007). The low levels of P-p38 in NQO1KO MEFs could be a mechanism to prevent apoptosis, as an attemp to uncouple the p38-dependent pathway. In addition to its role in apoptosis, a necessity for p38 has been also described for contact inhibition in a p38KO MEFs model (Faust, Dolado et al. 2005). We speculate with the idea that the lack of p38 can be partly responsible of the high cell saturation density of the NQO1KO cells. Similarly, previous results obtained by different groups (including ours) have demonstrated that the NQO1 expression depends on the cell culture confluency in normal adherent and tumor cells. NQO1 was increased at high cell density in BALB/c 3T3 clls (Schlager, Hoerl et al. 1993), human osteoblasts (Collin, Lomri et al. 2001) and HeLa cells (Bello, Gomez-Diaz et al. 2001, Bello, Gomez-Diaz et al. 2004). NQO1 activity and expression is also significantly increased in cell culture of human colon carcinoma HT-29 cells (Phillips, de la Cruz et al. 1994). NQO1 overexpression at high cell density could be linked to the cell cycle arrest in confluency. Accordingly, ROS levels are decreased in confluent fibroblasts and this decrease of ROS could be related to the increase in NQO1 levels which is also observed under these conditions (Pani et al., 2000). Thus, both the absence of NQO1 and the lack of p38 activation could account for an impairement in the contact inhibition leading to the higher saturation density.

A number of similarities exist between the processes of reprogramming and carcinogenesis. Cancer cells have a number of defined characteristics including sustained proliferative signaling and infinite replication (Hanahan and Weinberg 2000). Stem cells also possess this intrinsic ability for both self-renewal and proliferation, highlighting their similarity to cancer cells. Furthermore, studies from a number of cancers have shown that not all cells can regenerate tumors upon injection into immunodeficient mice, a functional assay which is now used to identify cells termed cancer stem cells (CSCs) (Beck and Blanpain 2013). In fact, transcription factors which play a critical role in reprogramming cells to pluripotency have also been identified in human cancers (reviewed by (Curry, Moad et al. 2015)).

In the case of the transcription factor Sox2, this is contained within lung and esophageal squamous cell carcinoma (SCC), being SOX2 identified as an oncogene for these cancers

(Bass, Watanabe et al. 2009). Sox2 is also expressed in both mouse and human pre-neoplastic skin lesions and SCCs, but not in normal epidermis. Deletion of SOX2 in melanoma or SSC caused regression of tumours, and also helped to identify a number of genes involved in proliferation, sternness and cell survival which are regulated by Sox2, providing further evidence of an important role for Sox2 in certain carcinogenesis processes (Boumahdi, Driessens et al. 2014). The higher levels of Sox2 in brain and liver of NQO1KO mice in relation to the wild-type counterparts could explain higher levels of tumor incidence upon deficiency of NQO1 that have been reported in the literature (Ross and Siegel 2004) and higher susceptibility to induced carcinogenesis (Long, Waikel et al. 2000, Long, Waikel et al. 2001).

In accordance, injection of NQO1KO MEFs into nude mice led to the development of teratomes, whereas wild-type MEFs were inactive. The latter is consistent with previous results that indicate that wild-type MEFs do not generate tumors (Maekawa, Shinagawa et al. 2007).When we examined the slices of these teratomas in the light microscope, several mesodermic cells and tissues (fibroblasts-like cells, white and brown adipose tissue, muscle) were readily observed, highlighting the proliferative and differentiation potential of MEFs lacking NQO1. In addition, these teratomes also contained areas of cells showing pyknotic nuclei, which is an indication of high levels of apoptosis. All together, our observations could indicate that the deletion of NQO1 in MEFs increases their tumorigenic potential. These cells exhibit high proliferation rates despite high apoptosis levels. They present high levels of Nrf2 nuclear translocation and an enhancement of the Akt/Nrf2 pathway. They show a decrease in the activation of the stress kinases p38 and JNK1. They are able to produce teratoma-type tumors and seem to retain certan pluripotency.

3. Concluding remarks

Taken together, our results indicate that, although Nrf2 and NQO1 participate in the same antioxidant signaling axis (Rushmore, Morton et al. 1991), the deletion of either Nrf2 or NQO1 generates very different cellular phenotypes. Nrf2KO MEFs evidenced a shortening of their life span in long-term cultures with lower proliferation rates. Conversely, NQO1KO MEFs exhibited a lengthened life span in long-term cultures, with higher proliferation rates. We hypothesize that the main growth differences of Nrf2KO and NQO1KO MEFs could be due to the Nrf2 status. In the case of Nrf2KO cells, these cells have suffered the total ablation of Nrf2 transcription factor. However, NQO1KO MEFs present the opposite phenotype, characterized by enhanced Nrf2 nuclear translocation. We propose this increased Nrf2

nuclear translocation as a hormetic response elicited to compensate the lack of the antioxidant enzyme NQO1.

However, it is true that both Nrf2KO and NQO1KO also share also some phenotypic features as high levels of apoptosis, even though certain mechanistic differences can be found. On one hand, NQO1KO MEFs present activation of both caspase-dependent and caspase-independent apoptosis pathways. On the other hand, Nrf2KO only showed an activation of the caspase-independent pathway, with the caspase-dependent pathway being blocked, at least partially. Treatment with a XIAP inhibitor unblocked caspase activation and triggered cell death in MEFs lacking Nrf2 but not in wild-type cells. Another similarity between both genotypes is the presence of alterations in mitochondria ultrastructure. Both Nrf2KO and NQO1KO MEFs exhibit smaller mitochondrial profiles but a higher portion of cytoplasm is occupied by these organelles. In relation to mitochondrial dynamics the situation is alike. Fission process does not seem to be affected in any of the two KO genotypes, with levels of Fis1 and Drp1 being unaltered. However, Nrf2KO MEFs showed lower levels of the fusion protein Mfn2. NQO1KO MEFs also showed decreased levels of Mfn2 and, in addition, of Opa1, another fusion protein,. When we measured the mitochondrial potential using the JC-1 probe, we also found divergences between both genotypes. We observed no changes among Nrf2KO and wild-type MEFs. Conversely in the case of NQO1KO, the cell population exhibited a lower mitochondrial potential. Therefore, we can conclude that both genotypes seem to have a mitochondrial network dysfunction, although NQO1KO MEFs exhibit a more striking phenotype. This is consistent with a role for the p62/Nrf2/NQO1 axis in the maintenance of mitochondrial integrity. Of note, NQO1 has been proposed as the most important element of this signaling cascade, playing a direct effector role, still to be characterized, to maintain mitochondrial integrity (Kwon, Han et al. 2012).

Deregulation of mitogenic pathways could surpass this increase of apoptotic signaling in MEFs lacking NQO1, resulting in the overall stimulation of growth rate and in the extension of long-term survival. We propose that activation of Nrf2 and Akt (with the subsequent increase in the glucose transporter Glut1), which are interrelated *via* a positive feedback loop, play a prominent role in determining the particular phenotype of NQO1KO MEFs, characterized by increased cell growth rates and long-term survival.

The function of Nrf2 in maintaining long-term viability of the MEFs and its role as a key factor for determining life span in *in vitro* and *in vivo* models (Sykiotis and Bohmann 2008, Leiser and Miller 2010, Singh, Niemczyk et al. 2010, Lerner, Bitto et al. 2013) may be also of high relevance for cancer research. In this way, upregulation of Nrf2 has been observed in

some cancers, where increased constitutive levels of Nrf2 protect cells to undergo apoptosis (Wang, Sun et al. 2008). In this sense, Nrf2 has been proposed as a good target for chemoprevention (Ganan-Gomez, Wei et al. 2013). We already know that homeostasis is essential for a healthy Nrf2 function. On one hand, Nrf2-mediated gene expression can play a protective role in order to avoid mutagenic processes. In this sense, expression of a Nrf2-dependent pleiad of antioxidant and preventive proteins is essential for removing carcinogens and to maintain cellular redox homeostasis. On the other hand, Nrf2 is overexpressed in a number of cancers. Once cells have undergone tumorigenesis, high levels of Nrf2 constitute a bad prognosis in cancer, because it can lead to chemoresistance of the tumor cell, and its protection against apoptosis (Lau, Villeneuve et al. 2008). Therefore, Nrf2 can be also a target for pharmacological inhibition in cancer. The use of inhibitors such as retinoic acid receptor α agonists that binds to Nrf2 in the newly designated Neh7 domain (Wang, Liu et al. 2013) or brusatol (Olayanju, Copple et al. 2015) could be a novel therapeutic tool, even though many more studies still need to be carried out. Inhibition of Nrf2-dependent pathways would favor cell growth arrest and sensitize cancer cells to undergo apoptosis (Wang, Sun et al. 2008, Suzuki, Motohashi et al. 2013). However, a point of caution should be taken because the role played by Nrf2 on cell transformation, like a double-edged sword, is cell lineage specific and depends on the cellular context (Jodar, Mercken et al. 2011).

Regarding NQO1, it is well-established that the polymorphism *NQO1*2* is a robust predictive factor in breast cancer (Fagerholm, Hofstetter et al. 2008) and a marker for the prognosis of hepatocellular carcinoma (Chiu, Ko et al. 2009). In addition, *NQO1* polymorphisms are associated with an increased risk of all types of leukemia (Bolufer, Barragan et al. 2006). Also, we have to consider the fact that the 4% of Caucasians and approximately the 20% of the Asian are homozygous for *NQO1*2* (reviewed by (Ross and Siegel 2004)), being a portion of the population potentially functional NQO1 knockouts. The fact that tissues and cells with the *NQO1*2* polymorphism exhibit no NQO1 activity and contain no detectable NQO1 polypeptide makes NQO1KO MEFs an interesting *in vitro* model to study the mechanisms underlying proliferation and apoptosis alterations in cells lacking NQO1 function.

Conclusions

- Nrf2 deletion increases basal apoptosis in a long-term cultures of MEFs and in Nrf2KO mice. Higher basal apoptosis in Nrf2KO MEF is related with increased levels of pro-apoptotic factors and proceeds through a caspase-independent mechanism, although the caspase cascade can be re-activated by XIAP inhibition.
- Regulation of Bcl-2 through the MAPK p38 is Nrf2-dependent. The kinase p38 is a negative effector of Bcl-2 function in wild-type MEFs through the inhibition of Nrf2 transcriptional activity.
- MAO-B accounts, at least partially, for the increased basal apoptosis levels of the Nrf2KO MEFs though the permeabilization of the OMM.
- Genetic deletion of Nrf2 or NQO1 causes alterations in mitochondrial ultrastructure and fusion/fission dynamics in MEFs.
- NQO1KO MEFs show higher levels of basal apoptosis through both caspase-independent and caspase-dependent apoptosis pathways.
- NQO1KO MEFs and NQO1KO mice exhibit higher Nrf2 nuclear translocation, which may have been developed as a hormetic response to compensate for the lack on the antioxidant enzyme NQO1.
- Despite their increased basal apoptosis levels, MEFs lacking NQO1 also show increase proliferative and tumorigenic potential, both *in vitro* and *in vivo*.
- Although Nrf2 and NQO1 participate in the same antioxidant signaling axis, genetic deletion of either Nrf2 or NQO1 generates very different cellular phenotypes in MEFs.

Conclusiones

- La deleción de Nrf2 incrementa la apoptosis basal en cultivos de MEFs y en ratones Nrf2KO. Estos niveles elevados de apoptosis en los MEFs Nrf2KO están relacionados con el incremento de factores pro-apoptóticos que actúan a través de una ruta independiente de caspasas, aunque la cascada de caspasas puede ser reactivada mediante la inhibición de XIAP.
- La regulación de Bcl-2 a través de la MAPK p38 es dependiente de Nrf2. La quinasa p38 es un regulador negativo de la función de Bcl-2 en MEFs silvestres a través de la inhibición de la actividad transcripcional de Nrf2.
- La actividad MAO-B es la causa, al menos en parte, del aumento de la apoptosis basal en los MEFs Nrf2KO a través de la permeabilización de la membrana mitocondrial externa.
- La deleción genética de Nrf2 o de NQO1 causa alteraciones de la ultraestructura mitocondrial, así como de los procesos de fisión y fusión mitocondrial en los MEFs.
- Los MEFs NQO1KO muestran unos mayores niveles de apoptosis basal mediante mecanismos tanto independientes como dependientes de caspasas.
- Los MEFs y los ratones NQO1KO presentan una mayor translocación al núcleo de Nrf2, la cual ha podido ser desarrollada como una respuesta hormética para compensar la falta de la enzima antioxidante NQO1.
- A pesar del incremento de la apoptosis basal, los MEFs carentes de NQO1 muestran un potencial tumorigénico y proliferativo mayor, tanto *in vitro* como *in vivo*.
- Aunque Nrf2 y NQO1 forman parte del mismo eje de señalización antioxidante, la deleción genética de Nrf2 o de NQO1 genera fenotipos celulares muy diferentes en los MEFs.

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Appendix A: Loading Control of Western Blots

The following images correspond to the loading controls (ponceau S) of the western blots that appear in the Results section.

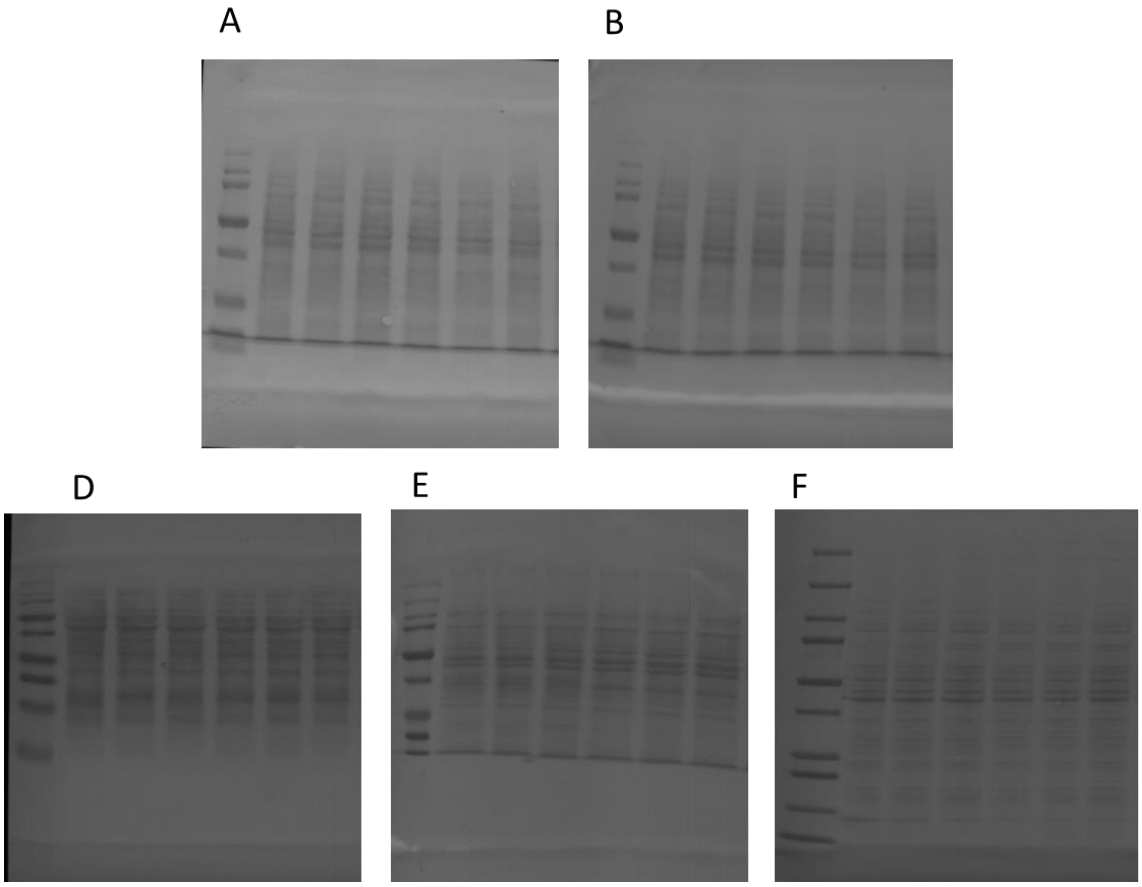

Figure 45: Ponceau S corresponding to Figure 15A, B, D, E and F, which describe markers of the mitochondrial apoptotic pathway in Nrf2KO MEFs.

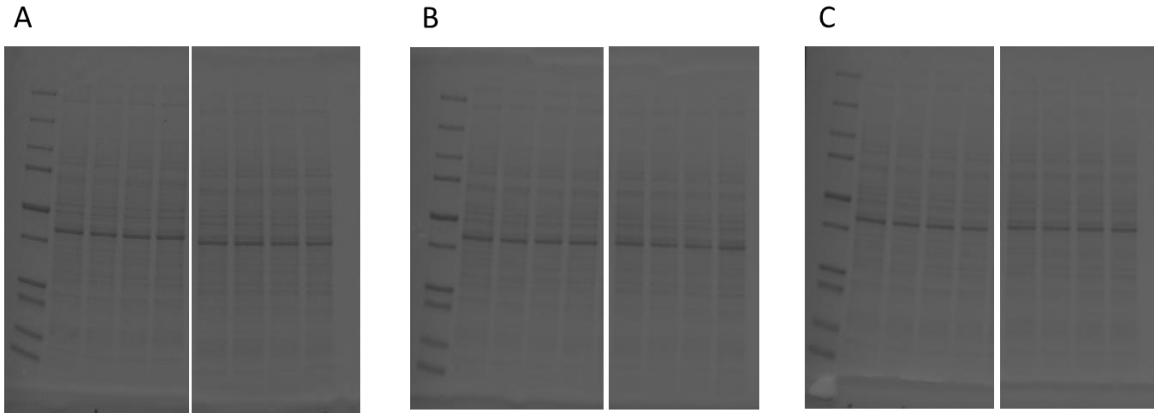

Figure 46: Ponceau S corresponding to Figure 18 and Figure 19, which describe the procaspases and cleaved caspases levels alterations in Nrf2KO MEFs. **A)** Corresponds to Figure 18A and Figure 19A. **B)** Corresponds to Figure 18B and Figure 19B and Figure 19C. **C)** Corresponds to Figure 18C and Figure 19D and Figure 19E.

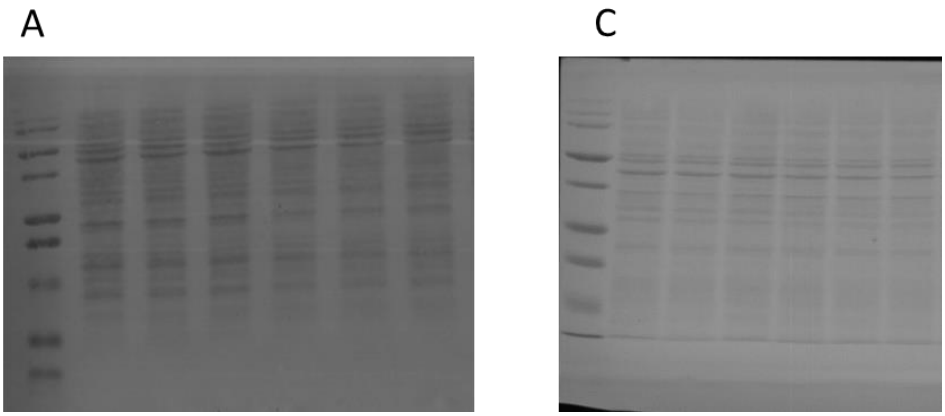

Figure 47: Ponceau S corresponding to Figure 20A and C, which describe the role of caspase-8 activation in Nrf2KO MEFs basal apoptosis and XIAP levels.

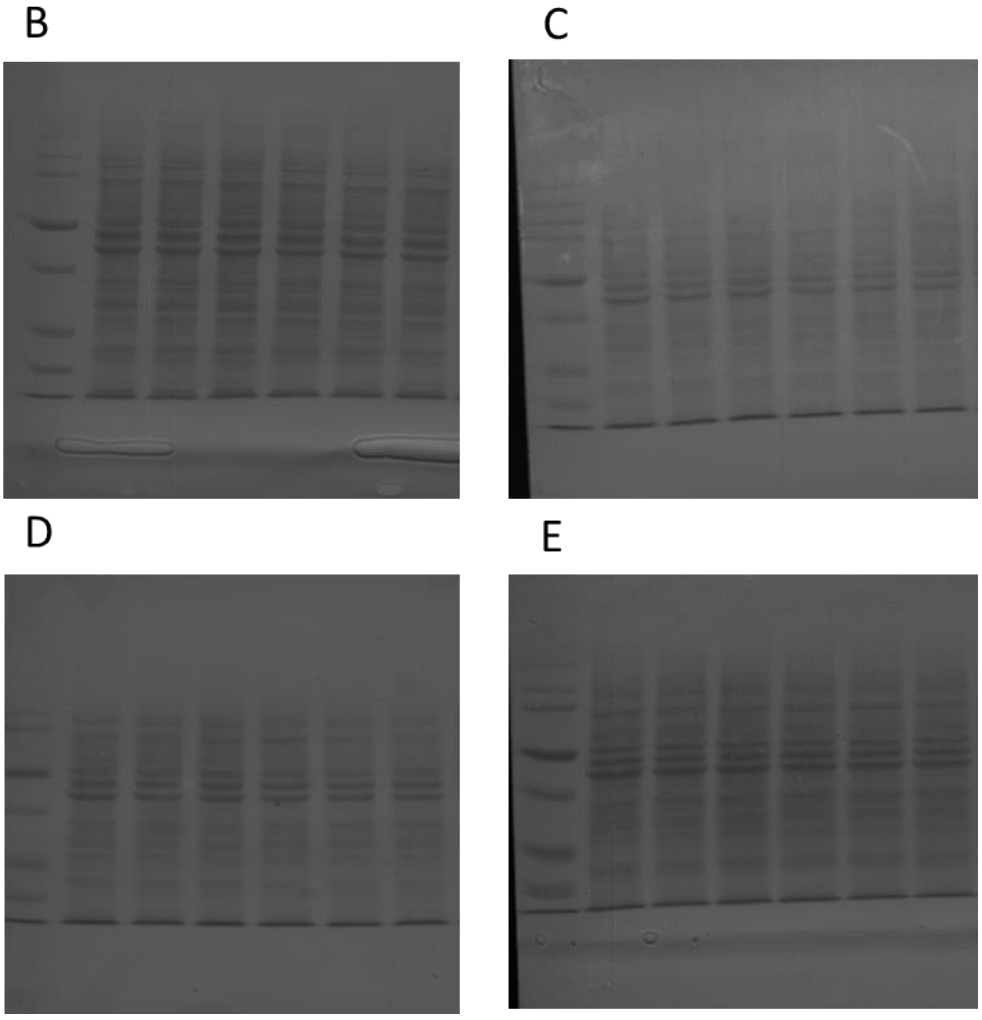

Figure 48: Ponceau S corresponding to Figure 21B, C, D and E, which describe alterations of MAPK and Akt pathways in Nrf2KO MEFs.

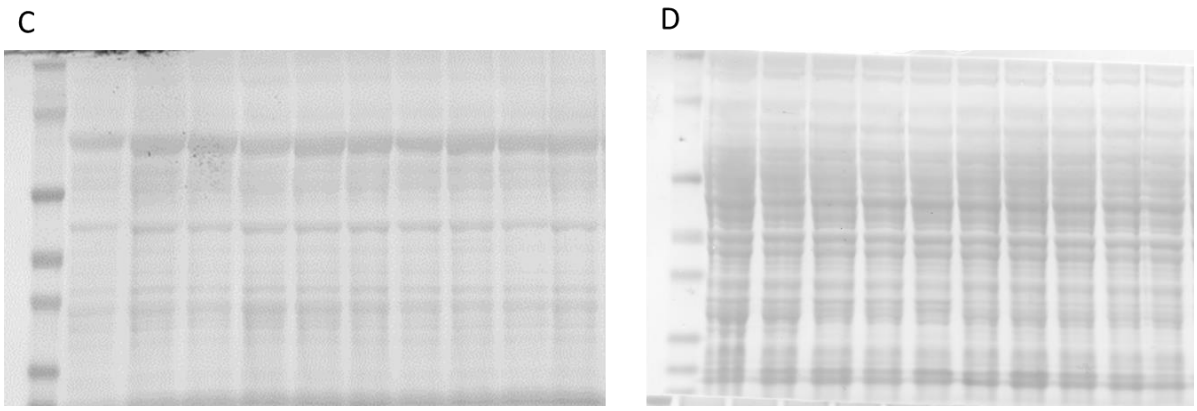

Figure 49: Ponceau S corresponding to Figure 22C and D, which describe the levels of p38 activation in liver and lung of Nrf2KO mice.

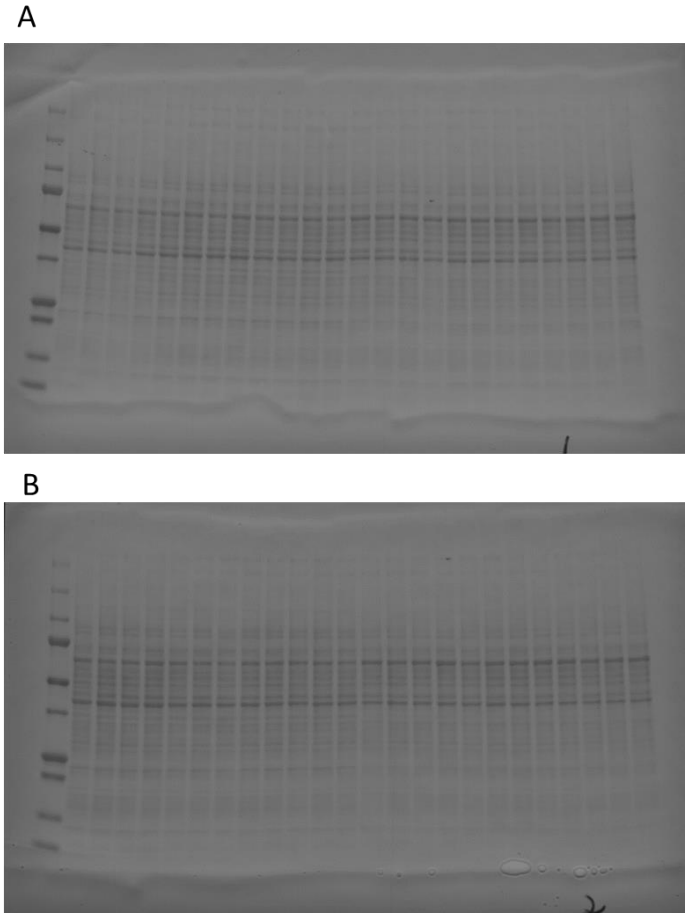

Figure 50: Ponceau S corresponding to Figure 23A and B,which describe Bcl-2 modulation by p38**. A)** Corresponds to the Bcl-2 western blot in Figure 23A and the Trx1 western blot in Figure 23B. **B)** Corresponds to the P-Bcl-2 western blot in Figure 23A.

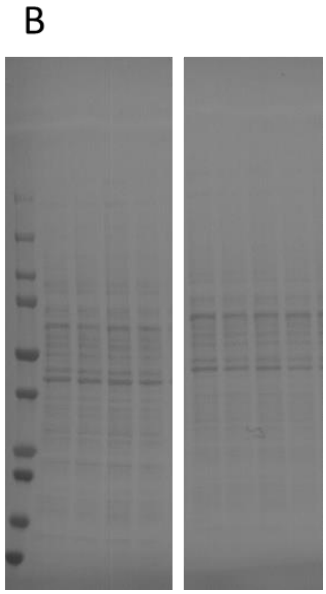

Figure 51: Ponceau S corresponding to Figure 24B, which describes the levels of MAO-B in Nrf2KO MEFs.

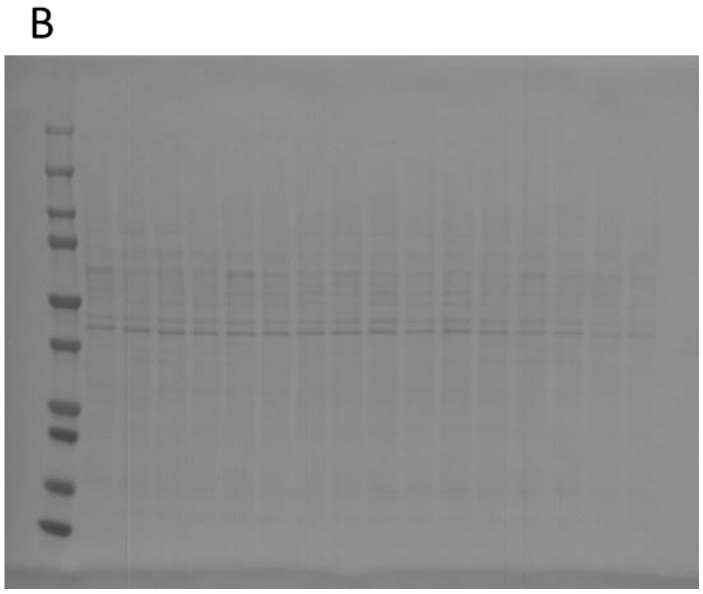

Figure 52: Ponceau S corresponding to Figure 25B, which describes the effects of XIAP inhibition in procaspases-3.

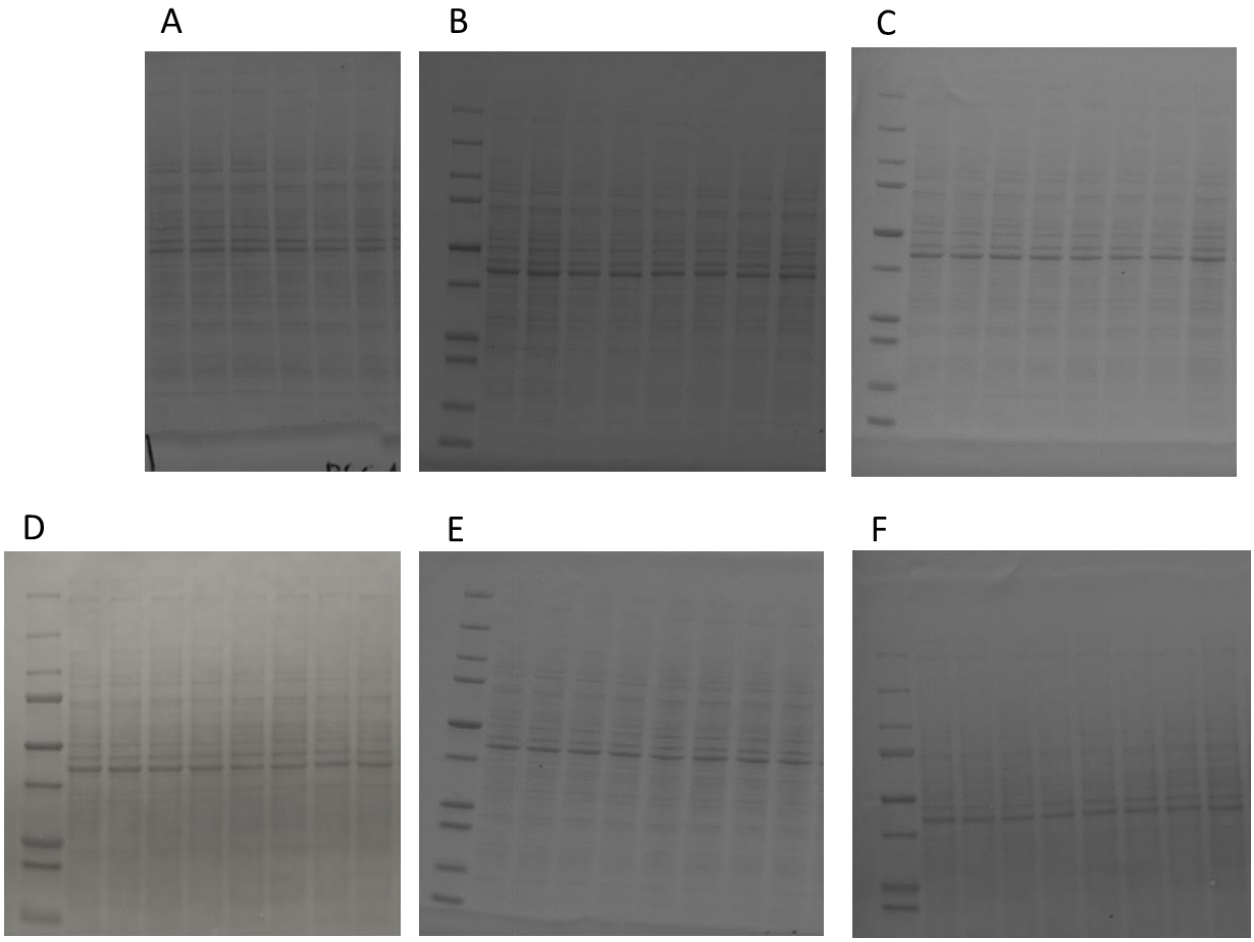

Figure 53: Ponceau S corresponding to Figure 27A, B, C, D, E and F, which describe alterations in mitochondrial fusion/fission markers in Nrf2KO MEFs.

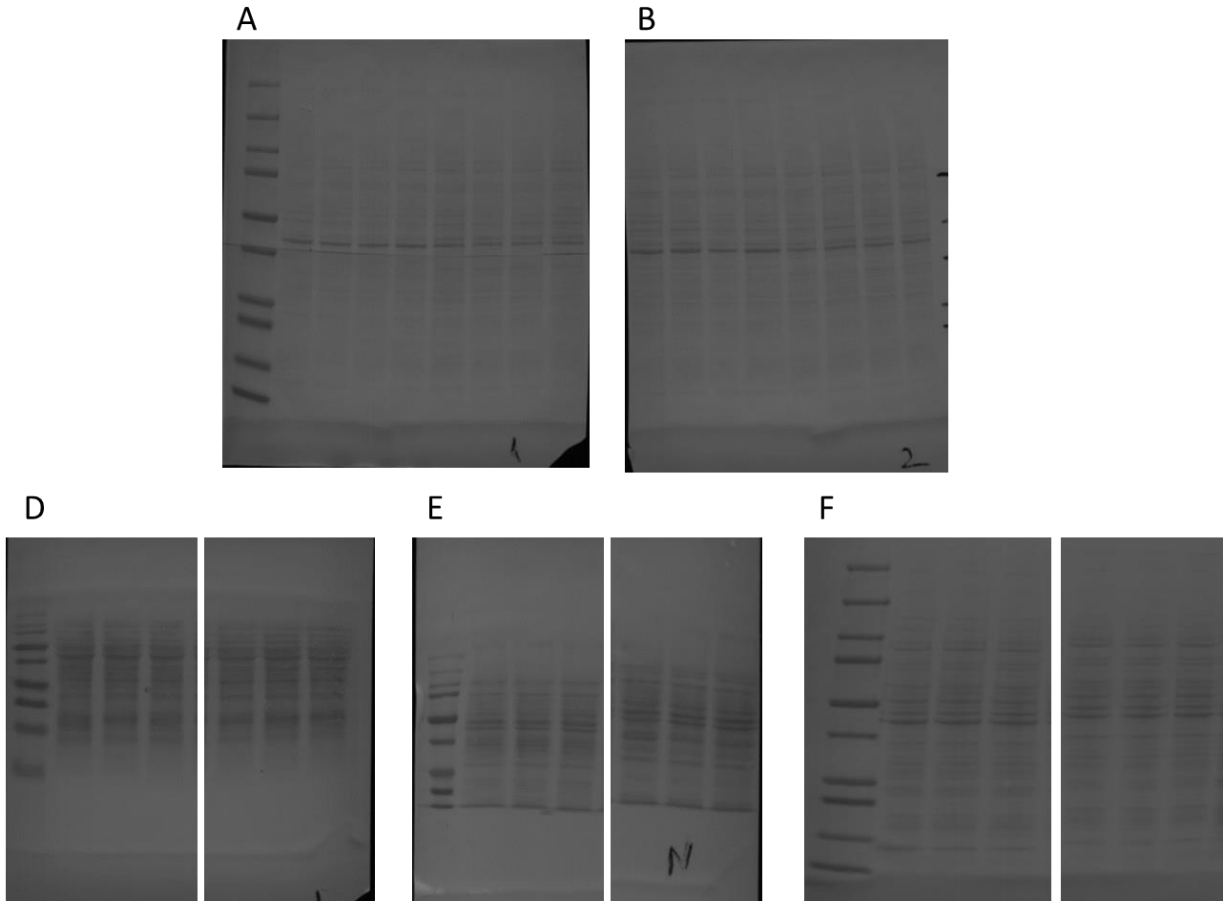

Figure 54: Ponceau S corresponding to Figure 31A, B, D, E and F, which describe mitochondrial pathway markers alterations in NQO1KO MEFs.

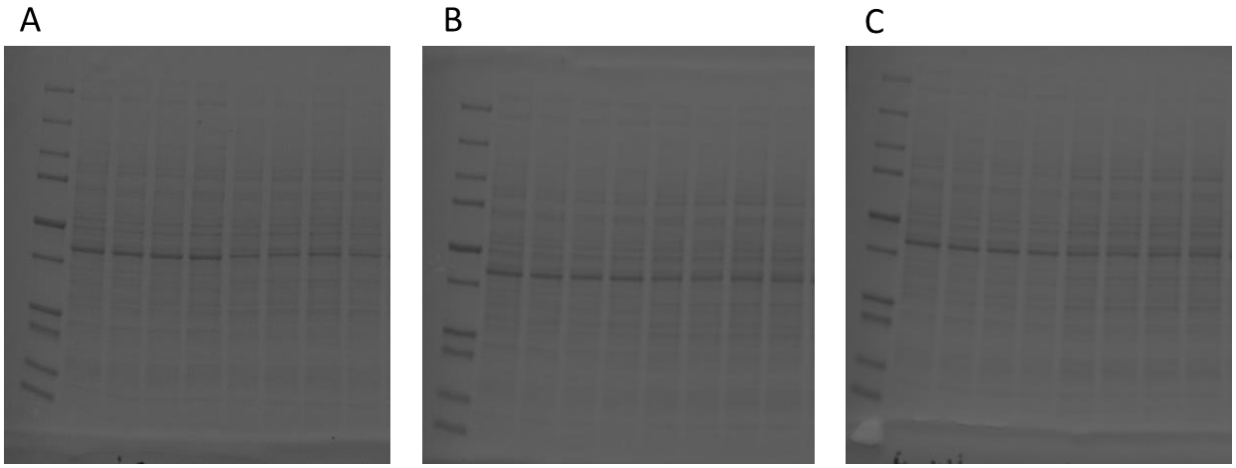

Figure 55: Ponceau S corresponding to Figure 34 and Figure 35, which describe procaspases and cleaved caspases levels. **A)** Corresponds to Figure 34A and 35A. **B)** Corresponds to Figure 34B and 35B and 35C. **C)** Corresponds to Figure 34C and 35D and 35E.

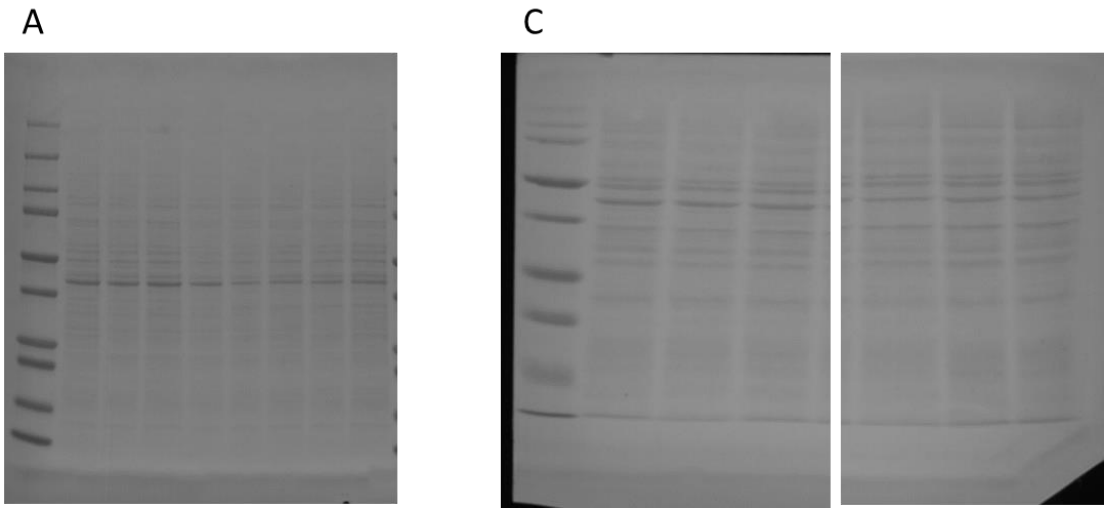

Figure 56: Ponceau S corresponding to Figure 36A and C, which describe the role of caspase-8 activation and XIAP levels in NQO1KO MEFs.

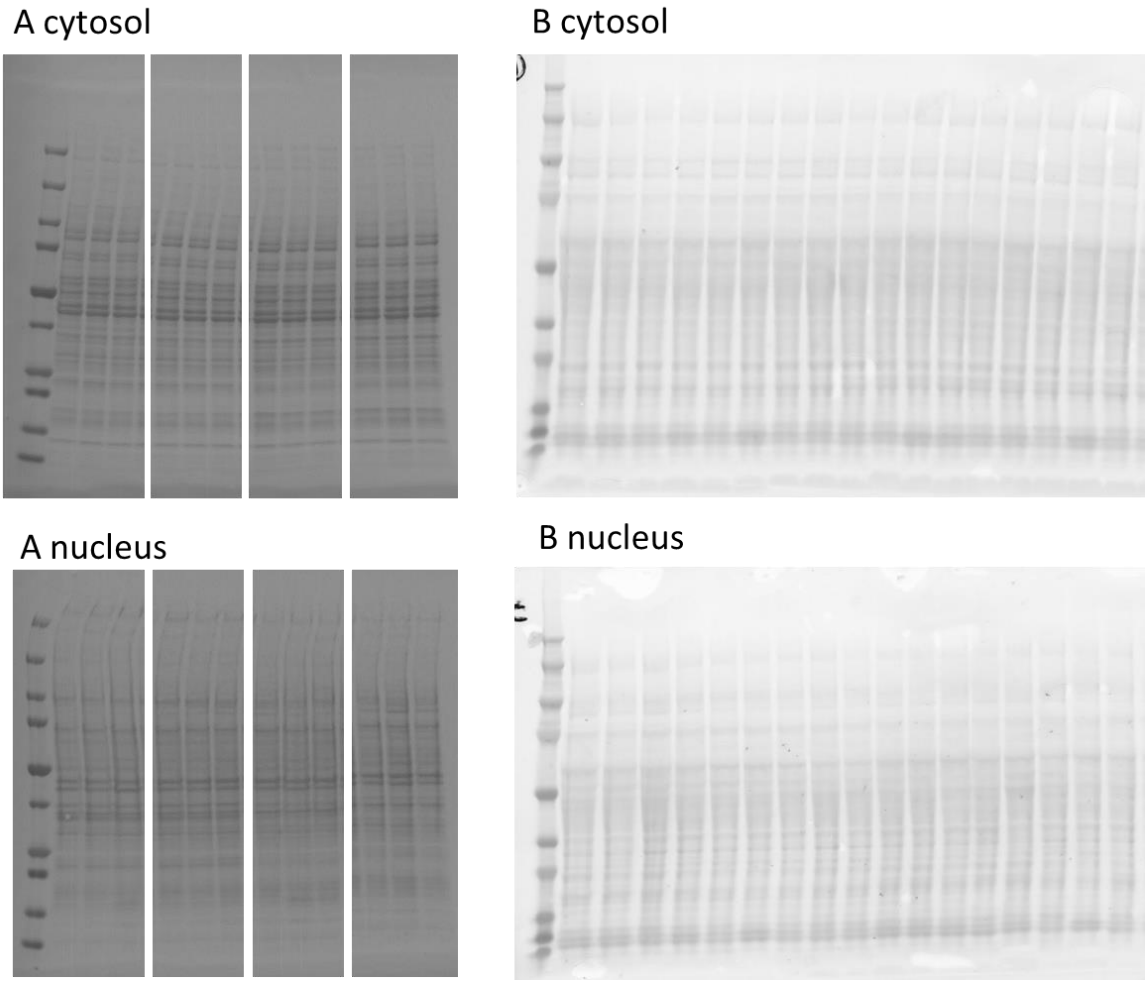

Figure 57: Ponceau S corresponding to Figure 37A and B, which describe Nrf2 nuclear translocation in NQO1KO MEFs and liver of NQO1KO mice.

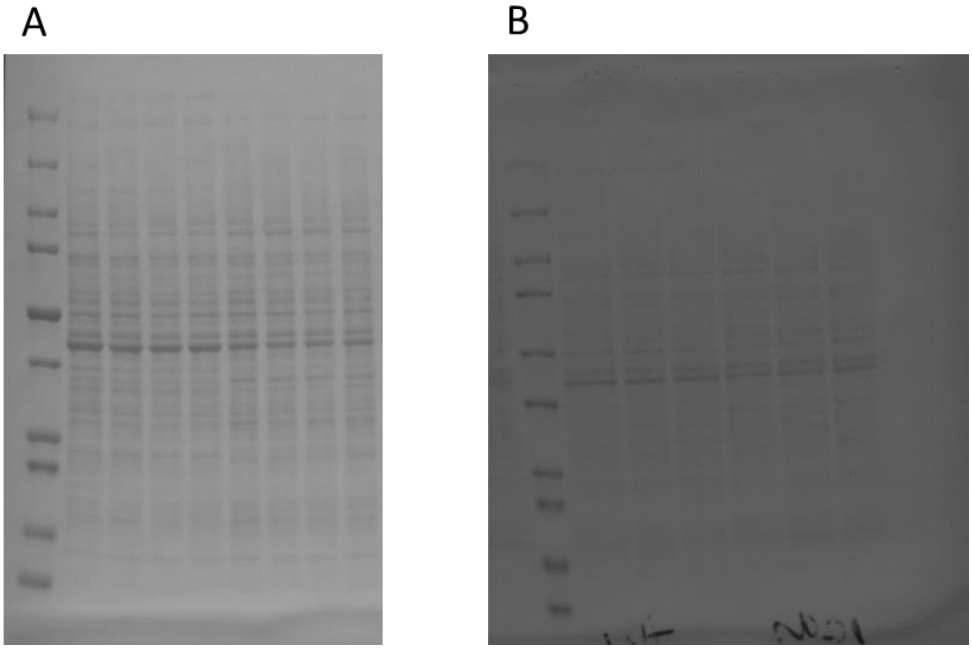

Figure 58: Ponceau S corresponding to Figure 38A and B, which describe Trx1 and p62 levels in NQO1KO MEFs.

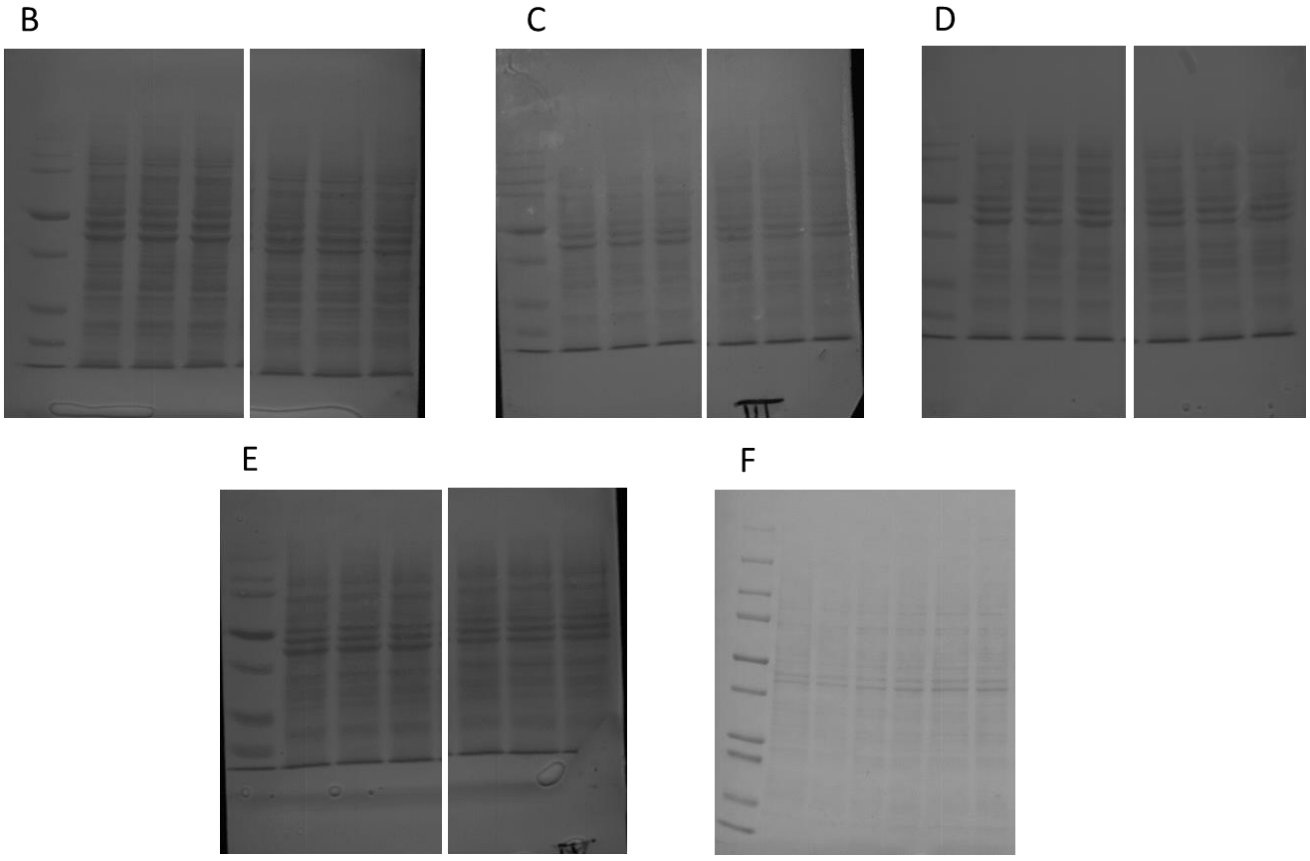

Figure 59: Ponceau S corresponding to Figure 39B, C, D, E and F, which describe alterations in MAPK and Akt pathways in the NQO1KO MEFs.

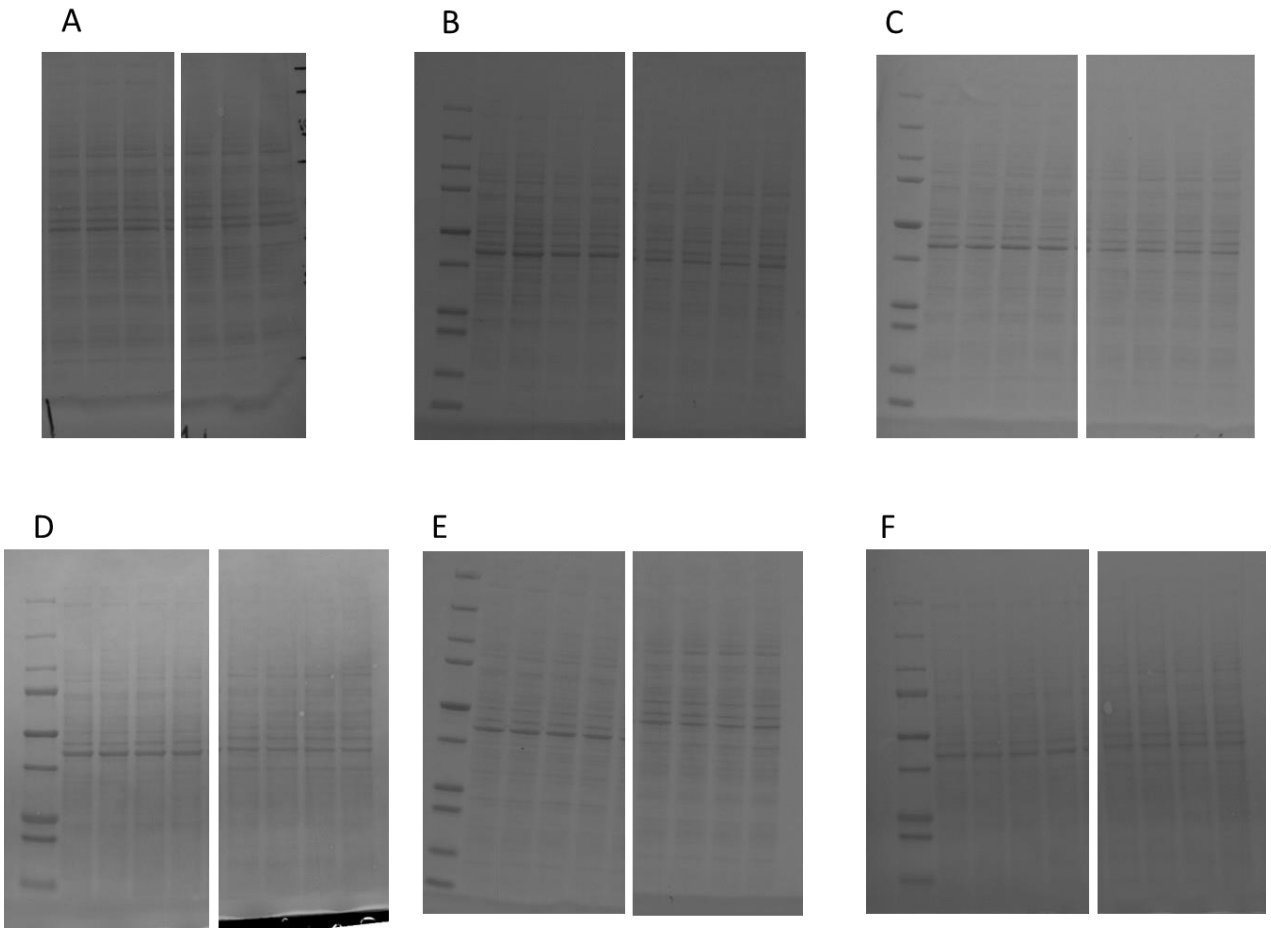

Figure 60: Ponceau S corresponding to Figure 41A, B, C, D, E and F, which describe alterations in mitochondrial fusion/fission markers in NQO1KO MEFs.

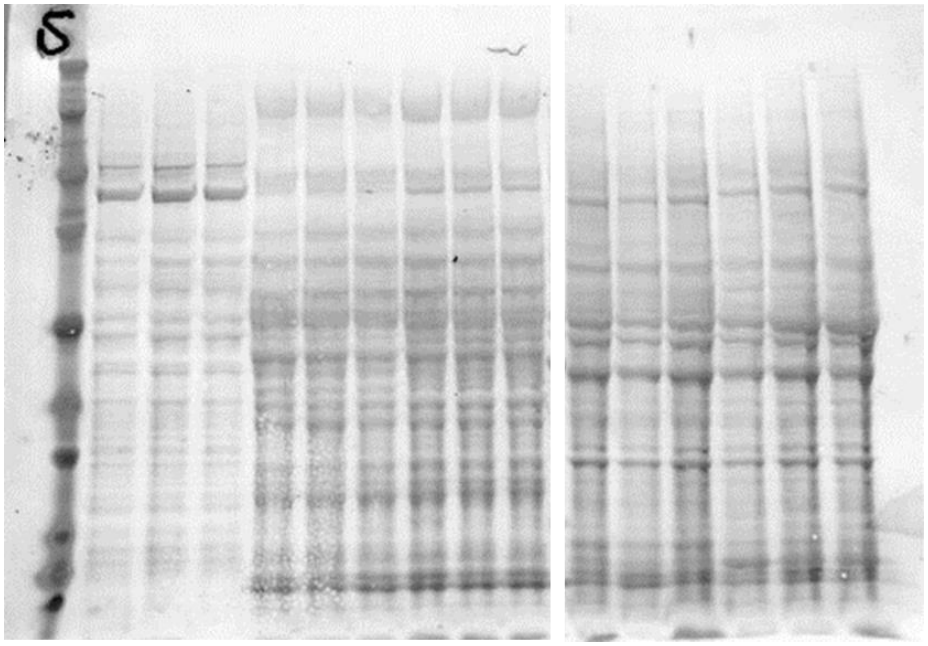

Figure 61: Ponceau S corresponding to Figure 42, which describes alterations in Sox2 levels in liver and brain of NQO1KO mice.