



FACOLTÀ DI SCIENZE MATEMATICHE FISICHE E NATURALI

Scuola Dottorale in Biologia
Sezione Biologia Applicata alla Salute dell'Uomo
XXV ciclo
(A.A. 2011/2012)

Response to DNA damage in terminally differentiated muscle cells

Risposta al danno al DNA in cellule muscolari terminalmente differenziate

Dottoranda:
Chiara Ferretti

Tutor:
Prof. Antonio Antoccia

Docente guida:
Dott.ssa Paola Fortini

Coordinatore: Prof. Paolo Visca

INDEX

RIASSUNTO	5
SUMMARY	9
I. INTRODUCTION	13
1. DNA damage	13
2. DNA repair capacity during cell differentiation	13
2.1 Homologous recombination and non-homologous end joining	13
2.2 Nucleotide excision repair	17
2.3 Base excision repair	20
2.4 Mismatch repair	22
3. DNA damage signalling	24
3.1 DNA damage response in post-mitotic cells	26
4. DNA damage and cell death	27
4.1 Apoptosis	27
4.2. Autophagy	29
II. RESULTS	33
III. DISCUSSION	53
IV. CONCLUSIONS	59
V. REFERENCES	61
ACKNOWLEDGEMENTS	77

RIASSUNTO

Nel muscolo scheletrico, il mantenimento e l'integrità del tessuto sono garantite da cellule staminali adulte, note come cellule satelliti, localizzate sotto la lamina basale (Shi X. et al., 2006). In questo studio cellule satelliti (mioblasti) di topo sono state indotte a differenziare *in vitro* in miociti mononucleati che raggiungono il differenziamento terminale fondendo in miotubi multinucleati. Questo modello è stato utilizzato per analizzare la capacità di riparazione del DNA e la DNA Damage Response (DDR) dopo trattamento con agenti genotossici in cellule muscolari post-mitotiche. Il sistema di differenziamento *in vitro* ben ricapitola il processo *in vivo* come dimostrato dall'uscita irreversibile dal ciclo cellulare, dalla repressione di geni associati alla proliferazione cellulare e dall'espressione di geni specifici del tessuto muscolare.

Studi precedenti condotti nel nostro laboratorio hanno dimostrato una forte riduzione nell'efficienza di riparazione del danno al DNA tramite il sistema di riparazione per escissione di basi (BER) in cellule muscolari post-mitotiche rispetto alla loro controparte proliferante (Narciso L. et al., 2007). A livello molecolare questa de-regolazione è dovuta alla down-regolazione di XRCC1 e alla mancanza della DNA ligasi I, componenti chiave dello short- e del long-patch BER, i due sub-pathways di tale via di riparazione. La ridotta capacità riparativa si traduce nell'accumulo di lesioni al DNA dopo esposizione a perossido di idrogeno, rilevate tramite il test della cometa e corrispondenti a una più lenta defosforilazione dell'istone 2AX.

Questo studio inizia con un'ulteriore caratterizzazione del BER tramite l'analisi dell'espressione genica di altri enzimi chiave quali le DNA glicosilasi, responsabili del riconoscimento e della rimozione di basi specifiche danneggiate od erroneamente incorporate nel DNA. I risultati mostrano una forte down-regolazione, nelle cellule muscolari terminalmente differenziate, di *Ung2* e *Myh* che codificano rispettivamente per l'uracile DNA glicosilasi 2 (UNG2) e l'adenina DNA glicosilasi (MYH). La de-regolazione di UNG2 e MYH è in linea con il ben noto coinvolgimento di questi due enzimi nella rimozione di lesioni che si formano durante la replicazione del DNA. La down-regolazione di UNG2 è stata confermata a livello funzionale tramite saggi di attività.

In linea con questi risultati ed estendendo il nostro studio ad altri tipi di danno, abbiamo osservato che in seguito ad esposizione a metil-metan-sulfonato (MMS), un agente alchilante le cui lesioni sono substrato per il BER, i miotubi accumulano nel DNA rotture a singolo filamento (SSB) e mostrano l'attivazione della DDR tramite fosforilazione dell'istone 2AX, rafforzando quanto osservato dopo danno ossidativo. Sorprendentemente, nonostante il danno accumulato a carico del DNA, le cellule muscolari post-mitotiche sono estremamente resistenti alla citotossicità indotta da una varietà di agenti che producono SSB. Abbiamo inoltre osservato che la fosforilazione dell'istone 2AX, successiva all'induzione di SSB, nei miotubi è ampiamente ATM-dipendente. Tuttavia, la cascata di segnalazione a valle di ATM è difettiva, come indicato dalla mancata

stabilizzazione e fosforilazione di p53, fenomeni che, come atteso, si osservano dopo trattamento dei mioblasti proliferanti. Pur stabilizzando p53 con la nutlina non si osserva morte cellulare nei miotubi dopo esposizione ad agenti genotossici che inducono SSB, indicando che tale resistenza sia dovuta ad un difetto di segnalazione a valle di p53. Al contrario, quando i miotubi vengono trattati con la doxorubicina (un inibitore della topoisomerasi2) e con il menadione (un inibitore della DNA polimerasi mitocondriale) la risposta citotossica è paragonabile a quanto osservato nei mioblasti ed è accompagnata dalla stabilizzazione e attivazione di p53. Nei miotubi l'ulteriore stabilizzazione di p53 con la nutlina determina, come atteso, un aumento dell'effetto citotossico indotto dalla doxorubicina, mentre l'uso dell'inibitore del proteasoma, MG132, attenua la letalità da essa indotta. Questi dati sono in linea con il meccanismo citotossico proposto per questo inibitore della topoisomerasi 2 che prevede l'esposizione delle rotture a doppio filamento solamente dopo rimozione degli "addotti enzima-DNA" tramite degradazione proteasomale. In netto contrasto, l'uso di MG132 incrementa la morte cellulare dopo trattamento con menadione, impedendo probabilmente la degradazione di p53. Esperimenti analoghi sono stati condotti in cellule muscolari derivate da topi privi di p53. I risultati hanno mostrato che la via di morte cellulare indotta dalla doxorubicina e dal menadione è strettamente p53-dipendente. Inaspettatamente, nessuna induzione di Bax e p21, geni normalmente attivati da p53, è stata osservata nelle cellule post-mitotiche dopo esposizione a questi agenti, lasciando aperta la domanda su quale fosse il meccanismo di morte cellulare attivato dai miotubi.

La nostra attenzione si è focalizzata sull'autofagia, un processo fisiologico deputato alla degradazione di proteine a emivita breve e di organelli e componenti cellulari danneggiati. Studi recenti indicano che l'autofagia è importante per il differenziamento cellulare, ma il suo ruolo in risposta al danno al DNA è meno chiaro. Per studiare il contributo dell'autofagia nella morte/sopravvivenza dei miotubi dopo stress genotossico, abbiamo prima di tutto analizzato il livello basale di autofagia durante il processo di differenziamento muscolare. Dal momento che l'autofagia è un processo dinamico, abbiamo utilizzato inibitori della funzionalità lisosomale che impediscono il completamento del processo autofagico portando all'accumulo del marcatore di membrana degli autofagosomi/autolisosomi, noto come LC3 II. Tale approccio sperimentale ha dimostrato chiaramente l'induzione dell'autofagia durante il differenziamento muscolare, sia come accumulo di LC3 II che come degradazione di p62, un altro marcatore di autofagia la cui digestione è testimonianza di un regolare flusso autofagico. Sorprendentemente abbiamo trovato che mTOR, il regolatore negativo dell'autofagia, è mantenuto attivo durante il differenziamento, suggerendo che il processo autofagico che accompagna il differenziamento muscolare sia indipendente dall'inattivazione di mTOR, analogamente a quanto osservato in cellule muscolari immortalizzate C2C12 (Tanida I. et al., 2006). Per verificare le eventuali conseguenze sul differenziamento muscolare dell'attivazione dell'autofagia dipendente

dall'inattivazione di mTOR, abbiamo indotto il differenziamento muscolare in presenza di rapamicina, un noto inibitore di mTOR. Tale trattamento non comporta alcun cambiamento nei livelli proteici di marcatori del differenziamento quali miosina (MHC) e desmina. Si osserva invece, come atteso, un aumento nell'induzione dell'autofagia, dimostrato dall'incremento dei livelli LC3 II e dalla diminuzione di p62. Nell'insieme questi dati suggeriscono che, sebbene il processo autofagico dipendente dall'inattivazione di mTOR sia funzionale nelle cellule muscolari, non è questa la via responsabile dell'autofagia basale che affianca il processo di differenziamento.

Nel corso di questo studio abbiamo inoltre osservato che durante il differenziamento muscolare si assiste a un riarrangiamento dell'espressione genica che determina la down-regolazione di p53 e di altri geni coinvolti nella DDR. Dal momento che la degradazione di p53 è stata descritta come necessaria per l'induzione di autofagia (Tasdemir E. et al., 2008), abbiamo testato l'effetto della stabilizzazione di p53 sia sul differenziamento muscolare che sul processo autofagico. La stabilizzazione di p53 con la nutlina non influenza il processo di differenziamento e non interferisce con l'induzione di autofagia. Si può anche escludere un ritardo nel flusso autofagico, in quanto la degradazione di p62 si verifica sia in presenza che in assenza di nutlina. Inoltre, l'autofagia si induce anche durante il differenziamento di cellule p53^{-/-} escludendo un contributo di p53 nella regolazione dell'autofagia basale di cellule muscolari.

Infine, per indagare se l'autofagia sia necessaria per il completamento del programma miogenico, abbiamo down-regolato *Beclin 1*, uno dei geni chiave dell'autofagia, tramite small interfering RNA (siRNA). I nostri risultati indicano che il silenziamento di *Beclin 1* durante il differenziamento muscolare induce cambiamenti sia a livello del differenziamento che della risposta autofagica. In miotubi down-regolati per *Beclin1* si osserva infatti sia una ridotta espressione di MHC che un ridotto accumulo di LC3 II. Inoltre, la down-regolazione di *Beclin 1* causa una stabilizzazione di p62 indicando un blocco nel flusso autofagico. È interessante notare che l'assenza della proteina Beclin 1 sembra interferire non con l'efficienza di differenziamento (nuclei appartenenti a cellule MHC positive/nuclei totali), quanto piuttosto con la capacità dei miociti di fondersi in miotubi (nuclei all'interno dei miotubi/numero dei miotubi totali) suggerendo che l'autofagia possa essere coinvolta in questa fase del processo di differenziamento muscolare.

Rimane ancora da chiarire se l'autofagia sia implicata come meccanismo di sopravvivenza che contribuisce alla resistenza delle cellule post-mitotiche in seguito a stress genotossico. In questo senso il processo autofagico potrebbe rappresentare una strategia volte al mantenimento dell'omeostasi e dell'integrità del tessuto muscolare. La caratterizzazione dei processi di morte cellulare dopo induzione di danno e dell'autofagia basale nelle cellule muscolari terminalmente differenziate potrebbe contribuire a fare luce sui meccanismi coinvolti nell'insorgenza di diverse miopatie, come la Distrofia Muscolare di Duchenne (DMD), che presentano accumulo di organelli danneggiati, tra cui anche

mitocondri. Molto recentemente l'autofagia è stata proposta come target terapeutico per la DMD (Pauly M. et al., 2012; De Palma C. et al., 2012) che potrebbe limitare il danno alle fibre muscolari promuovendo lo smaltimento degli organelli difettivi e riducendo i processi infiammatori.

SUMMARY

In skeletal muscle, tissue maintenance and repair are ensured by adult stem cells (satellite cells) that reside under the basal lamina (Shi X. et al., 2006). In this study mouse satellite cells (myoblasts) were induced to differentiate *in vitro* into mononucleated myocytes which fuse into multinucleated myotubes. This model was used to analyse DNA repair capacity and DDR after genotoxic insults in post-mitotic muscle cells. By genome-wide analysis it has been confirmed that the *in vitro* differentiation system completely recapitulates the *in vivo* process being characterised by irreversible cell cycle withdrawal, repression of cell proliferation-associated genes and expression of muscle-specific genes.

It has been described that Base Excision Repair (BER) efficiency is reduced in post-mitotic muscle cells when compared to their proliferating counterpart (Narciso L. et al., 2007). This impairment can be ascribed to the lack of DNA ligase I and the downregulation of XRCC1. Consistently, myotubes accumulate DNA lesions, detected by comet assay and H2AX phosphorylation, upon hydrogen peroxide exposure.

We carried out a further characterization of BER, analysing the gene expression of several DNA glycosylases, the enzymes involved in the first step of BER. Interestingly, we found that *Ung2* (uracil-DNA glycosylase2) and *Myh* (human mutY homolog) were clearly downregulated in post-mitotic muscle cells in agreement with the involvement of these two enzymes in the removal of lesions formed during S-phase. The activity of UNG2 was also impaired in myotubes as demonstrated by a functional assay with a specific substrate, further confirming a clear downregulation of this cell cycle-associated DNA glycosylase in terminally differentiated cells.

Extending our analysis to different DNA damaging agents, we observed that the exposure of myotubes to methyl methanesulfonate (MMS), a single strand break (SSB)-inducer, led to DNA damage accumulation which was mirrored by phosphorylation of H2AX. Surprisingly, we found that post-mitotic muscle cells were extraordinarily resistant to the killing effects of a variety of SSB-inducers despite the accumulation of DNA damage. We demonstrate that, upon SSB induction, H2AX phosphorylation occurs in myotubes and is largely ATM-dependent. However, DNA damage signalling cascade downstream of ATM is defective as shown by lack of p53 increase and phosphorylation. The stabilization of p53 by nutlin-3 was ineffective in activating the cell death pathway indicating that the resistance to SSB inducers is due to defective p53 downstream signalling. Conversely, doxorubicin (a Topoisomerase2 inhibitor) and menadione (an inhibitor of the mitochondrial DNA polymerase) were able to activate p53 and to kill myotubes suggesting that specific type of damage are required to induce cell death. We show that cell killing is p53-dependent although a restriction of p53 activated genes is observed.

In order to gain insights into the cell death pathways active in post-mitotic muscle cells, experiments were carried out to characterise autophagy as a

cellular stress response. Recent evidence indicate that autophagy is important for cell differentiation but its role in response to DNA damage is less clear. To investigate the contribution of autophagy in cell death or survival of myotubes upon genotoxic stress, we first analysed the basal level of autophagy during muscle differentiation. Since autophagy is a dynamic process, we exploited the use of lysosomal inhibitors which prevent degradation of the autophagic marker LC3 II. We observed accumulation of LC3 II and degradation of p62, a specific substrate for autophagy, as myoblasts differentiated into myotubes. As expected p62 degradation was affected by lysosomal inhibitors. Surprisingly, we found that mTOR, the negative regulator of autophagy, was not inactivated suggesting that during *in vitro* muscle differentiation autophagy is induced and is independent of mTOR inactivation accordingly with findings in C2C12 cells (Tanida I. et al., 2006).

To further characterize the autophagic pathway, rapamycin, a well known mTOR inhibitor, was added during myoblasts differentiation. No effect was observed on the expression of myosin heavy chain (MHC) and desmin, markers of muscle differentiation. Rapamycin increased autophagy induction as shown by the increase in LC3 II levels and by the decrease of p62.

Thus, we found that the mTOR inactivation-dependent autophagic pathway is functional in muscle cells although is not the pathway underlying the basal autophagy and is not involved in differentiation.

We demonstrated that during differentiation a significant gene reprogramming occurs and involves p53 downregulation in myotubes compared to myoblasts. Since it was described that p53 degradation is required for autophagy induction (Tasdemir E. et al., 2008), we tested the effect of p53 stabilization by nutlin-3 on both muscle differentiation and autophagy. We observed that p53 stabilization did not affect the differentiation process and did not interfere with the induction of autophagy. We can also exclude a delay in the autophagic flux since degradation of p62 occurred both in the presence and in the absence of nutlin-3. Moreover, we found that autophagy was also triggered during differentiation in a p53 null background indicating that p53 had no role in autophagy induction in murine muscle cells.

Finally, to investigate if autophagy was required to myogenic program, we downregulated *Beclin 1*, one of the key autophagic genes, by small interfering RNA (siRNA). Interestingly, the silencing of *Beclin 1* reduced both the protein expression of MHC and the accumulation of LC3 II compared to non-silenced myotubes. Moreover, *Beclin 1* siRNA induced a lack of p62 degradation indicating a block in the autophagic flux. Although the autophagy impairment observed, the phosphorylation of the mTOR target S6 was not affected indicating that mTOR was still active. Interestingly, the efficiency of differentiation, (MHC positive nuclei/total nuclei) was not influenced by *Beclin 1* downregulation whereas the fusion index (nuclei inside myotubes/number of myotubes) was reduced suggesting that autophagy could be involved in this step during muscle differentiation process.

Whether autophagy is implicated as a pro-survival mechanism in the resistance of post-mitotic cells to different genotoxic insults is currently under investigation. It is possible that the autophagic process could act as a strategy to maintain tissue homeostasis and integrity.

The comprehension of the mechanisms underlying the cell death pathway and the characterization of basal autophagy in muscle cells may contribute to shed light on the process involved in several myopathies, such as Duchenne Muscular Dystrophy (DMD). Indeed, very recent studies identify autophagy as a potential therapeutic target for ameliorating DMD (Pauly M. et al., 2012; De Palma C. et al., 2012) by removal of defective mitochondria and inhibition of muscle damage and inflammation.

I. INTRODUCTION

1. DNA DAMAGE

Maintenance of genetic stability is crucial to ensure tissues preservation and homeostasis during the lifespan. Alteration of DNA sequence can lead to expression of non functional protein limiting cellular performance or can cause the activation of oncogenes and/or the inactivation of oncosuppressor genes inducing uncontrolled cell proliferation. The biological effects of the persistence of DNA damage depend on the occurrence of the lesion in somatic or germinal cell lineages and are represented by tumors, premature aging and genetic diseases.

DNA is constantly subjected to genotoxic stress due to the exposure to environmental agents, such as UV light, ionizing radiation (IR) and chemical pollutants, or to endogenous compounds generated as by-products of oxidative metabolism. Depending on the time maintenance and function of a specific cell type the risk of accumulating DNA damage may vary. For instance damage to proliferating/stem cells if not repaired can lead to mutation amplification or propagation through the processes of self-renewal and differentiation, respectively whereas damage to post-mitotic cells can affect mostly tissue homeostasis. To counteract these effects cells are provided of a variety of DNA repair mechanisms and signalling pathways that ensure that DNA damage is repaired. Emerging evidence suggests that proliferating cells address DNA damage differently from their differentiated counterpart and this issue will be the subject of the next sections.

2. DNA REPAIR CAPACITY DURING CELL DIFFERENTIATION

Cells have evolved several mechanisms of DNA repair to ensure genome integrity: homologous recombination (HR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR).

Depending on cell-type, differentiation stage and age, cells may have different need to withstand genotoxic stress and therefore might have different priorities in the use of the various DNA repair pathways.

2.1 *Homologous recombination and non-homologous end joining*

Double strand breaks (DSB) represent the most lethal form of DNA lesion, so an accurate mechanism to repair DSB is required to preserve genome stability. Mammalian cells present two major pathways for repair of DNA DSB:

homologous recombination (HR) and non-homologous end joining (NHEJ) (reviewed by Bernstein K.A. and Rothstein R., 2009). In HR, an undamaged DNA strand containing hundreds of base pairs of sequence homology is used as a template to replace an adjacent damaged one. For this reason HR is a high-fidelity mechanism. Because sister chromatids are available as templates upon DNA replication, HR is active mainly in S/G₂ phases of the cell cycle and therefore it is precluded in non-cycling cells. In mammals the MRN complex (that consists of Mre11-Rad50-Nbs1) together with the DNA endonuclease CtIP does the initial processing of the DNA ends. Following resection, the exposed ssDNA is coated by replication protein A (RPA), which recruits the Rad52 epistasis group of proteins to enable Rad51 filament formation (Fig. 1). Then, Rad51 facilitates the formation of a physical connection between the invading DNA substrate and homologous duplex DNA template, leading to the generation of heteroduplex DNA (D-loop). Here, Rad51–dsDNA filaments are formed by accommodating both the invading and donor ssDNA strands within the filament. Finally, Rad51 dissociates from dsDNA to expose the 3'-OH required for DNA synthesis using the invading 3'-end as a primer. The breast cancer susceptibility genes BRCA1 and BRCA2 are also involved in facilitating orderly HR.

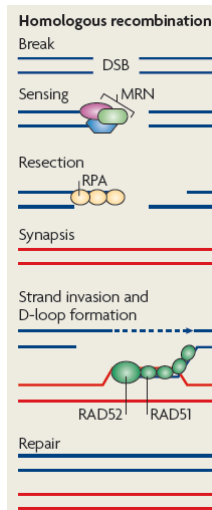


Figure 1. Homologous recombination repair. After the recognition of the DNA lesion, the MRN complex is recruited to DSB and generates single-stranded DNA (ssDNA) by resection. ssDNA is coated by RPA and bound by Rad51 and Rad52 leading to homologous template invasion and D-loop formation (modified from Misteli T. et al., 2009).

In contrast to HR, NHEJ requires little or no sequence homology for efficient repair and can be error-prone. This mechanism is active during all cell cycle phases and is the predominant pathway for DSB repair in mammalian cells. NHEJ results in minimal DNA end processing and requires at least seven proteins: Ku70, Ku80, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, X-ray cross complementing 4 (XRCC4), XRCC4-like factor (XLF) and DNA ligase IV (LIGIV) (reviewed by Lieber M.R. et al., 2008). The basic mechanism of NHEJ has been worked out in great detail (Fig. 2).

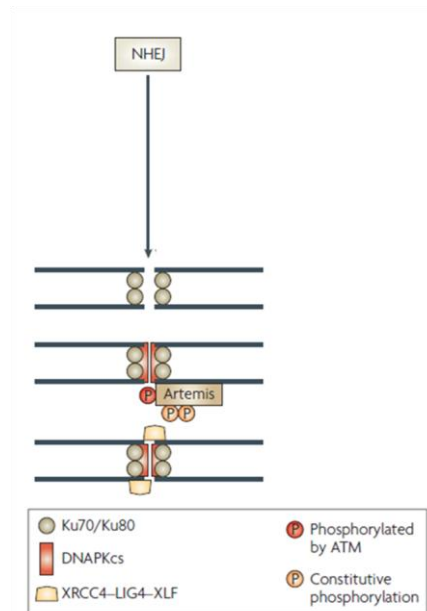


Figure 2. Players of non-homologous end joining. Ku70 and Ku80 form a heterodimer (Ku) which binds to and encircles broken DNA ends. Ku, besides protecting DNA ends from exonucleolytic attack, also recruits DNA-PKcs, a phosphoinositol-3-like family serine/threonine protein kinase. Together, Ku70, Ku80 and DNA-PKcs form the DNA-dependent protein kinase complex (DNA-PK) and the assembly of this trimeric complex on the ends of double-stranded DNA activates the kinase activity of DNA-PKcs. DNA-PKcs, in turn, phosphorylates and activates the nuclease Artemis, which facilitates “cleaning up” of the ends. As a final step, ligation of the broken ends is catalyzed by the trimeric LIG IV complex, which consists of the catalytic core, DNA ligase IV, and its two accessory factors, XLF and XRCC4 (modified from Löbrich M. et al., 2007).

Beside this canonical NHEJ, another mechanism named backup NHEJ (B-NHEJ) (Wang H. et al., 2003; Nussenzweig A. et al., 2007) or microhomology-mediated end joining (MMEJ) (McVey M. et al., 2008) has been described but the mechanism, the regulation and the factors involved in it remain elusive.

Defects in DSB repair lead to premature aging, neurodegeneration and increased cancer susceptibility. Mutations in the HR proteins BRCA1/2 that cause increased breast and ovarian cancers in women have been linked to the accumulation of genetically unstable mammary stem cells (SC) (Liu P. et al., 2008) and mutations in model mice defective in NHEJ components have been shown to display hematopoietic SC (HSC) self-renewal defects (Kenyon J. et al., 2007; Rossi D. J. et al., 2007).

DSB can be lethal to a cell and proliferating/stem cells present an efficient response to this type of damage. The basal level of γ -H2AX foci (a DSB marker) and the expression of several DSB repair genes, as BRCA1, XRCC5 and Rad51, are higher in embryonic stem cells (ESC) when compared to fibroblasts (Tichy E. D. et al., 2008; Maynard S. et al., 2008). Furthermore, ESC are suggested to repair DSB more quickly than mouse 3T3 cells after ionizing radiation exposure (Tichy E. D. et al., 2008).

Proliferating/stem cells mainly rely on HR rather than NHEJ for high-fidelity DSB repair. A comparative analysis of the DSB repair capacity of cycling and non-dividing cells in mouse shows that HR is the major mechanism to repair DSB in ESC with a minimal role played by NHEJ. In contrast, when ESC are induced to differentiate, NHEJ becomes the predominant pathway and HR is reduced. The different use of NHEJ seems to be due to the change of the protein level of DNA ligase IV during the differentiation process. Indeed, the abundance of ligase IV is low in ESC and increased in mouse embryonic fibroblasts (MEF) concomitantly with NHEJ activity (Tichy E. D. et al., 2010).

Also in human cells HR is the election pathway to repair DSB and its efficiency decreases when human embryonic stem cells (hESC) are induced to differentiate in neural progenitors and in astrocytes (Adams B.R. et al., 2010). Differently from mouse, hESC also utilize NHEJ, despite the preferential use of HR.

The selective requirement for HR or NHEJ during nervous system development has been analysed by an elegant study (Orii K. E. et al., 2006). By using mice carrying a germ line disruption of XRCC2 (HR defective) and DNA ligase IV (NHEJ defective) the two pathways for DSB repair are found to be spatio-temporally distinct: HR inactivation leads to abundant apoptosis in proliferating neural precursor cells and resultant lethality around embryonic days 9-10 (E9-E10), whereas the disruption of NHEJ has deleterious consequences only at later developmental stages (not before E12). Since the late stages of the embryogenesis are characterized by massive differentiation, these results suggest that the HR pathway has an essential protective role against the lethality of DSB in proliferating cells becoming dispensable in post-mitotic cells where NHEJ is the repair pathway of election. To further testify the key role of the NHEJ in differentiated long-lived cells a faster DSB repair kinetics in adipocytes compared to their proliferating precursors is found after exposure to a radiomimetic chemical or ionizing radiation (Meulle A. et al., 2008). In this case, the increased ability of adipocytes to repair DSB is mainly ascribed to the upregulation of DNA-PK expression and activity.

Interestingly, a recent study shows that the induction of NHEJ after H₂O₂ treatment (working concentration close to concentration in normal brain) in postmitotic neurons requires G₀ exit mediated by pRb phosphorylation directed by cyclin C. Abrogation of G₀-G₁ transition by silencing of cyclin C compromises NHEJ activation while forcing G₁ entry determines NHEJ induction even in absence of DNA damage (Tomashevski A. et al., 2010). So, the re-entering into the cell cycle to activate NHEJ can explain the expression of cell cycle markers observed in damaged neurons of normal brain (Schmetsdorf S. et al., 2009).

In the hematopoietic system, the induction of NHEJ in stem cells can be detrimental for genome stability. Hematopoietic development starts from largely quiescent, slowly cycling HSC that, in response to environmental stimuli, are able to give rise to a series of proliferating committed progenitors and mature cells. In contrast to human HSC (hHSC) that undergo apoptosis upon IR exposure, murine HSC (mHSC) survive and repair DNA damage by error-prone NHEJ that can lead to accumulation of gene aberrations. Differently, myeloid progenitors from mouse mainly die after IR treatment and surviving cells use high-fidelity HR (Naka K. et al., 2011; Mohrin M. et al., 2010).

2.2 Nucleotide excision repair

The removal of UV light-induced photoproducts, bulky chemical adducts and intra-strand DNA cross-links depends on nucleotide excision repair (NER). This mechanism consists of three sub-pathways, namely global genome repair (GGR), transcription-coupled repair (TCR) and domain-associated repair (DAR). While GGR operates on the entire genome, TCR and DAR participate in the repair of active genes. In particular, TCR provides efficient repair of the transcribed strand only and DAR repairs both strands of active genes and is detectable only when GGR is attenuated (Noussipiel T., 2009a).

NER is well characterised at mechanistic level and it is known that during GGR the double helix distortion caused by DNA damage is sensed by the heterotrimer XPC/HR23B/Centrin2, with the contribution of the DDB heterodimer for some lesions. In the next step a denaturation bubble is opened around the lesion by the transcription factor TFIIH and later the damaged strand is nicked by XPG on the 3' side of the lesion and by the heterodimer ERCC1/XPF on the 5' side. Finally, after the displacement of an oligonucleotide encompassing the lesion, the undamaged strand is used as a template to fill the gap. The TCR mechanism is very similar, differing only in the recognition step that involves RNA polymerase II (RNAP II) rather than XPC and DDB complexes (Fig. 3).

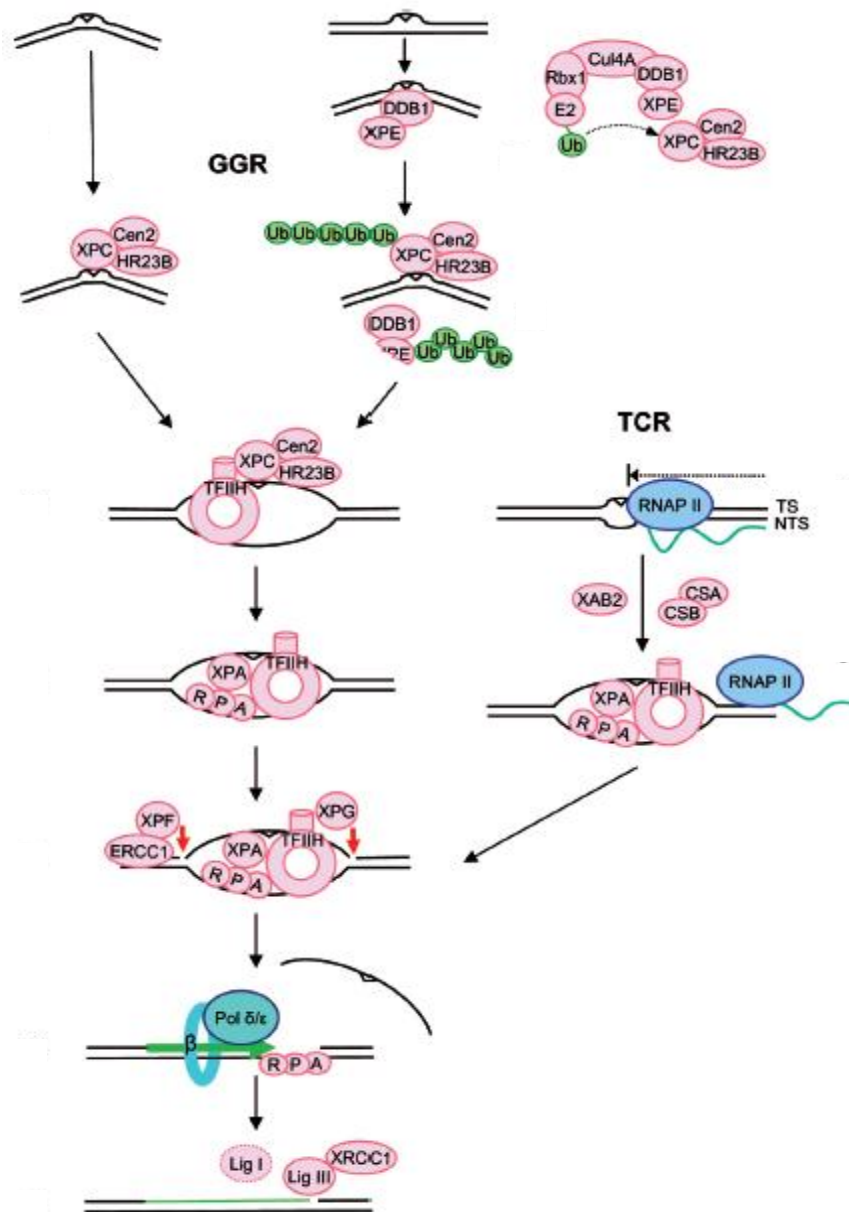


Figure 3. Sub-pathways of NER. In GGR the lesion sensing involves XPC/HR23B/Cen2 whereas in TCR DNA damage is recognized by RNAP II. Subsequently TFIIH opens a denaturation bubble around the lesion, XPG nicks at the 3' and ERCC1/XPF at the 5'. The resulting gap is filled by DNA polymerase δ or ϵ . The nick is sealed by ligase III with a minor contribution of ligase I in replicating cells (modified from Nouspikel T., 2009b).

CS proteins associate with UV-stalled RNAPII and are required for efficient TC-NER by recruiting NER factors and chromatin remodelers (Fousteri M. et al., 2008). The importance of functional NER is illustrated by genetic disorders like Cockayne syndrome (CS) and trichothiodystrophy (TTD) defective in TC-NER, and Xeroderma pigmentosum (XP) characterized by GGR defects or total NER deficiencies.

Pioneering studies carried out by Hanawalt and co-workers in several human terminally differentiated tissues, i.e. striated muscle, macrophages and neurons, show that NER is generally attenuated during cell differentiation. More precisely, in terminally differentiated cells GGR is lower than the undifferentiated counterpart whereas, within active genes, not only the transcribed, but also the non-transcribed strand are efficiently repaired (Nospikel T., 2007; Nospikel T., 2009a). At molecular level the attenuation of GGR could be ascribed to the incomplete phosphorylation of ubiquitin-activating E1 enzyme Ube1 upon differentiation that could lead to a reduction in the ubiquitination of TFIIH decreasing its activity in GGR (Nospikel T. et al., 2006). This phenomenon also occurs in human B lymphocytes in which NER at global level is downregulated (Hyka-Nospikel N. et al., 2011). B lymphocytes are G₀-arrested cells that retain the ability to re-enter the cell cycle upon a proper stimulus. Like terminally differentiated cells, in these quiescent B cells a reduction in phosphorylation of Ube1 and GGR impairment is observed, while transcribed genes are efficiently repaired by TCR and DAR. Interestingly, upon proliferation, an increase in phosphorylation of Ube1 and NER recovery is detectable. It is important to note that NER impairment during quiescence could lead to DNA damage accumulation at global genome level that would increase the likelihood of mutation fixation upon proliferative stimuli promoting B cell malignancies or abnormal immune functions (Hyka-Nospikel N. et al., 2011). The different contribution of NER sub-pathways in protection during differentiation is also detected analysing UV sensitivity of stem cells and differentiated cells (de Waard H. et al., 2008). UV sensitivity of MEF depends on the capacity of perform TC-NER, as shown by marked UV-sensitivity of *Xpa*^{-/-} and *Csb*^{-/-} MEF compared to GGR defective MEF. Conversely, GGR is the main determinant of UV sensitivity in ESC as shown by severe UV sensitivity of *Xpa*^{-/-} and *Xpc*^{-/-} ESC compared to *Csb*^{-/-} TC-NER defective ESC. Altogether these studies suggest that NER downregulation is a common feature in G₀-arrested cells, either quiescent or terminally differentiated. It is reasonable that non-dividing cells, which only have to maintain genomic integrity of transcribed genes and preserve tissue specificity, do not need a severe surveillance of the entire genome but an efficient repair of active genes. In contrast, ESC cannot rely on only TCR or DAR because the accumulation of damage and mutations in non-transcribed genes would be detrimental for a proper development and lead to genetic instability.

2.3 Base excision repair

Structurally non-distorting lesions in DNA, such as alkylated, oxidised bases and abasic sites are mainly processed by BER. The first step of BER implies the removal of the damaged base by specific DNA glycosylases which give rise to abasic sites rapidly converted into SSB by an AP endonuclease. After that, the polymerization step involves either the short patch BER (SP-BER) or the long patch BER (LP-BER) pathway that differ for the repair-patch length and the specific players involved (Fig. 4). DNA polymerase β and DNA ligase III are responsible for the filling-in and the sealing step of the SP-BER, respectively, whereas, in LP-BER, these reactions are catalysed by the PCNA-dependent DNA polymerases δ/ϵ and/or DNA polymerase β and DNA ligase I (Fortini P. et al., 2007).

BER is the repair mechanism of election for oxidative DNA damage that is also produced spontaneously as a consequence of aerobic metabolism. Post-mitotic tissues such as muscle and brain present high metabolism and therefore are particularly subjected to ROS-induced damage. In skeletal muscle the BER efficiency has been addressed by Narciso and coworkers using an *in vitro* murine system which recapitulates the *in vivo* differentiation process (Narciso L. et al., 2007). Muscle satellite cells (myoblasts) isolated from mouse thigh are adult stem cells that are able to proliferate and differentiate, in appropriate culture medium, in multinucleated myotubes. A comparative analysis of BER capacity in myoblasts versus myotubes shows a clear impairment of BER in post-mitotic muscle cells. Both the SP-BER and LP-BER sub-pathways are compromised although the LP-BER, which shares several partners with DNA replication, is more severely affected. At molecular level the BER impairment can be ascribed to the nearly complete lack of DNA ligase I and to the strong down-regulation of XRCC1, a scaffold protein known to be essential for DNA ligase III stabilization. Consistently, XRCC1 is a transcriptional target for FoxM1 and E2F1 which activate several cell cycle genes and are both downregulated upon cell cycle exit in myotubes (Fortini P. et al., 2010). In contrast, downregulation of XRCC1 is not observed in post-mitotic neural cells where this enzyme has a protective role against the cytotoxicity induced by oxidative DNA damage (Kulkarni A. et al., 2008). Moreover, XRCC1 seems to be important during neurogenesis since its inactivation leads to loss of cerebellar interneurons and abnormal hippocampal functions (Lee Y. et al., 2009).

Since the mitochondrial genome is a susceptible target for oxidative damage its repair should play an important role particularly in post-mitotic tissues with high metabolism. It has been recently discovered that repair in mitochondria occurs not only by SP-BER but also by LP-BER. The LP-BER is involved in the processing of oxidative lesions (Sung J. S. et al., 2005) and requires the nuclease activity of FEN1 to repair damage both in nuclear and mitochondrial genome (Liu P. et al., 2008; Szczesny B. et al., 2008). FEN1 is not only involved in LP-BER, but it is also essential for DNA replication.

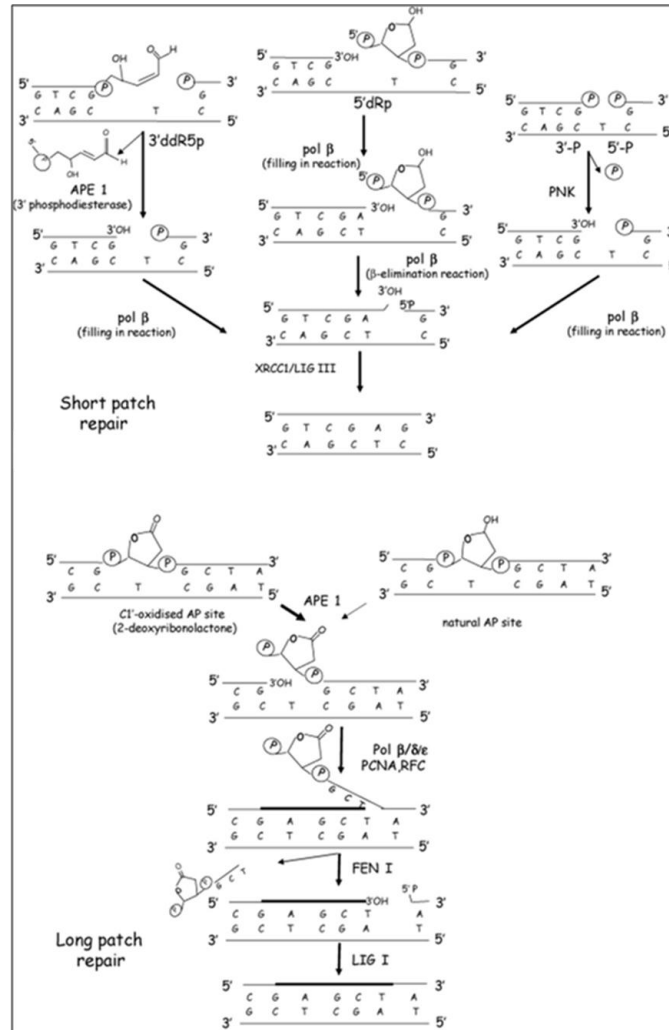


Figure 4. Schematic representation of short patch and long patch BER repair (Fortini P. et al., 2007).

FEN1 is strongly downregulated in terminally differentiated cells. How this might impact on the efficiency of mitochondrial BER in differentiated tissues under normal and pathological conditions is currently under investigation. A comparison of BER efficiency between stem and differentiated cells shows that the former have a higher BER activity and a faster repair after oxidative

stress compared to the latter one. BER proteins such as Ape1, DNA ligase III, PCNA, UNG2 and XRCC1, are expressed in murine ESC at higher level than MEF and this is also true for α OGG1 and Ape1 in hESC after H₂O₂ treatment (Tichy E. D. et al., 2011; Maynard S. et al., 2008).

If in general DNA repair is downregulated during differentiation a notable exception is represented by the immune system where the defective BER observed in monocytes is enhanced during dendritic cell maturation (Briegert M. et al., 2007). The BER defect in monocytes may account for their selective killing during tumor therapy with alkylating agents.

2.4 Mismatch repair

DNA mismatches can occur not only in proliferating cells but also in non-cycling cells because of base deamination or during repair attempts by error-prone DNA polymerases. The key proteins in MMR are highly conserved from bacteria to mammals: in eukaryotes there are multiple homologs of the key bacterial MutS and MutL MMR proteins (Harfe B. D. and Robertson S. J., 2000). Six human mismatch repair genes (MSH2, MSH3, MSH6, MLH1, PMS1 and PMS2) have been identified as components of this repair system that efficiently corrects single base mismatches and loops of one to three extrahelical nucleotides. MMR degrades the section of the error-containing strand (Fig. 5) and therefore provides the DNA polymerase with another chance to generate an error-free copy of the template sequence. MMR is also one of the alternative pathways involved in minimizing the toxic and mutagenic effects of the DNA 7,8-dihydro-hydroxyguanine (8-oxo-dG), a harmful DNA oxidation product causing G>T transversions and implicated in frameshift formation (Colussi C. et al., 2002). The microsatellite instability and the mutator phenotype of MMR-defective cells strongly support a crucial role of MMR in the DDR (Macpherson P. et al, 2005). Moreover, inherited MMR defects are associated with the hereditary nonpolyposis colorectal cancer (HNPCC), an autosomal dominant disorder.

The comparison of the MMR efficiency of dividing cells and differentiated cells is addressed by few studies. MMR has been reported to be active in differentiated neurons (Belloni M. et al., 1999) and as efficient as in undifferentiated cells (David P. et al., 1997; Panigrahi G. B. et al., 2005). In contrast, one report indicates that MMR proteins, namely MSH2 and MSH6, are highly expressed in mouse ESC and MMR is significantly more active in ESC than in MEF (Tichy E. D. et al., 2011).

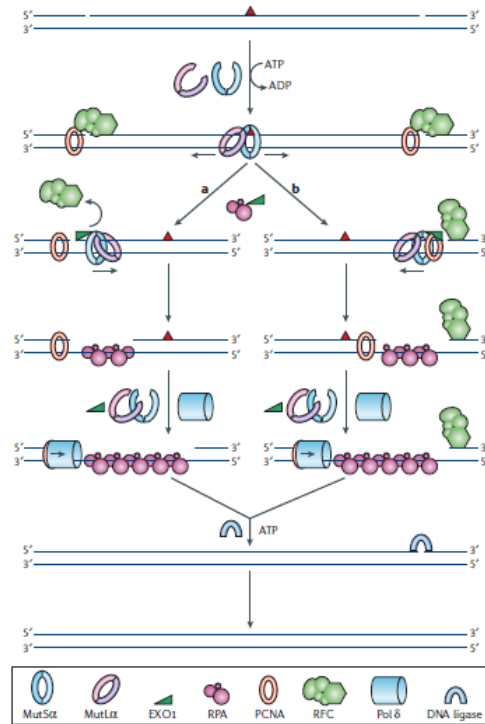


Figure 5. The reconstituted human mismatch-repair system. The mismatch (red triangle)-bound MutS α (or MutS β) recruits MutL α leading to the release of the sliding clamp from the site of the mismatch. **(a)** Clamps diffusing upstream displace RFC and load EXO1 which degrades the strand in a 5'→3' direction. RPA stabilizes the ssDNA and the gap is filled by PCNA-bound Pol δ . DNA ligase I seals the nick completing the repair process. **(b)** Clamps migrating downstream encounter a PCNA molecule bound at the 3' of the strand break. The RFC molecule that is bound at the 5' side of the break would not be displaced by MutS α , preventing degradation in the 5'→3' direction. The complex that contains MutS α , MutL α , EXO1, PCNA and RFC would assemble at the 3' of the nick and mediate degradation towards the mismatch (Jiricny J., 2006).

3. DNA DAMAGE SIGNALLING

DNA damage induces an evolutionary conserved signalling pathway defined as DNA damage response (DDR) that is a network of interacting pathways coordinating repair and cell cycle progression. The DDR involves damage sensors, signal transducers and effectors. DNA damage recognition by the sensor proteins starts a cascade that results in cell cycle arrest and DNA repair and can lead to cell death depending on the type of damage.

Localization of DDR factors to DNA damage sites requires special sensor proteins that directly recognize specific DNA lesions and activate the DDR. A group of proteins, including mediator of DNA damage checkpoint protein 1 (MDC1/NFBD1), 53BP1, Mre11-Rad50-NBS1 (MRN complex) and the Rad9-Rad1-Hus1 (9-1-1) complex have been implicated as DNA damage sensors and recognize DNA strand breaks as well as region of replication stress (Ward I. et al., 2004; Wang B. et al., 2002; Stucki M. et al., 2004; Paull T.T. et al., 2005). Binding of the MRN and 9-1-1 complexes to the sites of DNA damage in chromatin activates the signal transducing kinase ataxia-telangiectasia mutated (ATM), the ATM and Rad3-related (ATR) kinase, and DNA-PK, all of which are members of the phosphoinositide 3-kinase-related kinase family. The transducer kinases respond to different types of stimuli. DNA-PK and ATM are preferentially activated in response to DSB while ATR primarily responds to SSB and stalled replication forks. ATM and ATR are conserved serine/threonine kinases characterized by a C-terminal catalytic motif containing a phosphatidylinositol 3-kinase domain. They share many targets that are phosphorylated by ATM immediately after the appearance of DSB, and later by ATR, which probably maintains these phosphorylations for extended periods of time (Shiloh Y., 2001). Once activated, ATR and ATM amplify the damage signal by phosphorylating several substrates, including key DNA damage response proteins H2AX, BRAC1, NBS1, SMC1, MDC1 and 53BP1, as well as repair proteins Ku70/80, Artemis and Rad-like proteins (Pallis A. G. et al., 2010; Freeman A. K. et al., 2010). DSB and stalled replication forks cause the phosphorylation of histone 2AX at Ser139 (H2AX for the unphosphorylated form and γ H2AX for the phosphorylated form) *via* ATM and ATR respectively. For DSB the distribution of γ H2AX within the nucleus is localised to the DSB. These γ H2AX foci appear 10 min after DSB induction and over time their amount and intensity decrease due to the activity of phosphatase PP4 (Nakada S. et al., 2008; Chowdhury D., et al., 2008), testifying that DSB repair is occurring. The functional significance of γ H2AX is assumed to be a signal that facilitates the repair of free DSB or DSB formed at stalled replication forks, presumably by causing the chromatin to be more accessible for DNA repair.

In order to allow time for DNA repair, ATM and ATR temporarily arrest the cell cycle by phosphorylation and activation of cell cycle checkpoints. To this aim, important phosphorylation targets of ATM and ATR are Chk1 (checkpoint kinase-1), Chk2 (checkpoint kinase-2) and p53. ATM phosphorylates Chk2 after

DSB formation at Thr68 (Zhou B. B. et al., 2000; Matsuoka S. et al., 2000), while ATR phosphorylates Chk1 at Ser345 upon stalled replication forks (Liu Q. et al., 2000; Guo Z. et al., 2000). In turn, Chk2 and Chk1 phosphorylate the transcription factor p53 at Ser20 (Shieh S. Y. et al., 2000). Phosphorylation of p53 at Ser20 does not activate the transcription factor, rather it prevents the proteasomal degradation of p53. Under unphosphorylated conditions, p53 is ubiquitinated by the ubiquitin E3 ligase MDM2 (mouse double minute 2) which targets it for proteasomal degradation. Phosphorylation of p53 by Chk1 and Chk2 prevents this process and results in stabilization of the p53 protein (Chehab N. H. et al., 1999; Chehab N. H. et al., 2000; Hirao A. et al., 2000; Unger T. et al., 1999). ATM and ATR can also phosphorylate p53 at Ser15, thereby increasing its transactivation activity (Banin S. et al., 1998; Canman C. E. et al., 1998).

Upon activation, p53 transcriptionally upregulates the cyclin-dependent kinase inhibitor p21waf1/cip1, which suppresses cyclin E/CDK2 kinase activity and prevents G₁ to S phase progression (Deng C. et al., 1995). Phosphorylation of Chk2 or Chk1 by ATM and ATR respectively, leads to the phosphorylation and subsequent ubiquitination of Cdc25A, and in this way prevents the activation of CDK2, resulting in lack of DNA polymerase recruitment to replication origins and thereby blocking the transcription of genes required for S phase progression.

ATM and ATR also regulate the G₂/M cell cycle block. Chk1 phosphorylates Cdc25c (cell division cycle 25 homolog C), a dual specificity tyrosine and serine/threonine phosphatase, at Ser216 causing the binding of Cdc25c to the 14-3-3 σ protein (Peng C. Y. et al., 1997; Sanchez Y. et al., 1997). Within this complex, Cdc25c is transported out of the nucleus and is thereby unable to dephosphorylate/activate Cdk1, which finally results in G₂/M arrest (Dalal S. N. et al., 1999).

Once the process of DNA repair has been successfully completed, cells can re-enter the cell cycle, whereas irreparable DNA damage can lead to irreversible cell cycle arrest, known as senescence, or induction of cell death.

The critical role of DDR for overall tissue integrity and function is well illustrated by the severe clinical consequences observed in both humans and mice for mutations in genes regulating this pathway (Hakem R., 2008; Kerzendorfer C. et al., 2009). Patients with mutations in the kinase ATM present blood vessels abnormalities, cerebellar degeneration, immunodeficiency and increased cancer risk (Hoeijmakers J. H., 2009) whereas ATR mutations cause developmental defects in humans and mice (Hakem R., 2008; Hoeijmakers J. H., 2009; Seita J. et al., 2010).

3.3 DNA damage response in post-mitotic cells

If accumulation of damage occurs in post-mitotic cells it can also contribute to disease progression or lead to loss of tissue homeostasis. Conditions such as neurodegeneration and cardiomyopathy may arise as a consequence of damage in terminally differentiated cells. Whereas proliferating cells adopt the canonical DDR described above, terminally differentiated cells show alterations in the response to DNA damage. This issue has been largely addressed in the muscle and nervous system also because of the availability of well characterized *in vitro* cell systems suitable for molecular/biochemical characterization. DNA damage induced by the anthracycline antibiotic doxorubicin results in selective decrease in the expression of muscle-specific genes in rat cardiac muscle cells providing the first evidence that DNA damage can block cell differentiation (Ito H. et al., 1990). More recently, a differentiation checkpoint activated by DNA damage was identified in skeletal muscle progenitors that coordinates the repair and the expression of differentiation-specific genes during cell cycle arrest (Puri P.L. et al., 2002). Upon exposure to genotoxic stress the myogenic differentiation is reversibly inhibited while DNA is repaired. The transient phosphorylation of MyoD by DNA damage activated c-abl that inhibits MyoD-dependent transcription of muscle genes is likely to be the key event in the coordination of repair and differentiation. The mechanism of inhibition of muscle gene transcription depends on the cell cycle phase at which myoblasts arrest in response to damage (Simonatto M. et al., 2011). This is in striking contrast with what occurs in proliferating cells exposed to genotoxic agents that differentiate due to the p53-mediated repression of the stem cell marker Nanog (Lin T. et al., 2005).

DNA damage response of post-mitotic cells differs from that of cycling cells because several genes involved in DDR are also cell cycle-related genes that undergo downregulation during terminal differentiation. An example is provided by p53 that is involved in the regulation of differentiation and development. p53 transcripts reach a maximum during differentiation of several tissues but the levels decrease strongly in cells undergo terminal differentiation. In some species the absence of p53 during development leads to dramatic defects in differentiation (Wallingford J. B. et al., 1997). Other instances are represented by myotubes that are deprived of ATR and Chk1 but retain the ability to activate the ATM-Chk2 branch upon radiation damage (Latella L. et al., 2004). In post-mitotic neurons the response to DSB is mediated by ATM (Biton S. et al., 2006; Biton S. et al., 2007). Suppression of the function of ATM attenuates both apoptosis and cell cycle re-entry triggered by some genotoxic compounds (Kruman I. I. et al., 2004). The Chk1/Cdc25A axis participates in the activation of cell cycle-mediated neuronal death (Zhang Y. et al., 2006). A recent study provides evidence that terminally differentiated astrocytes lack functional DDR signalling (Schneider L. et al., 2012). The activation of ATM and its downstream DDR factors are highly inhibited due to repression at

transcriptional level. The transcriptional control is also responsible for the strongly impairment of the axis ATM-Chk2-p53 making astrocytes extremely resistant to irradiation. DNA-PK is actually responsible for IR-induced H2AX phosphorylation in astrocytes whereas in the neural stem cells ATM appears to be the main responsible kinase within a canonical DDR.

4. CELL DEATH

If DNA lesions persist after activation of DDR and DNA repair, proliferating cells can either arrest the cell cycle progression in G₁ (cellular senescence) or trigger programmed cell death pathways, such as apoptosis (self-killing) and autophagy (self-eating). Quiescent/post-mitotic cells cannot adopt the replicative senescence or the canonical cell cycle arrest-dependent apoptosis as strategies to get rid of damaged cells but they are provided of alternative strategies (e.g. autophagy).

4.1 Apoptosis

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Apoptosis is a well orchestrated mechanism developed by eukaryotic organisms during evolution process. It is also known as cellular self destruction or cell-suicide or programmed cell death (PCD) (Savitz S. I. et al., 1998). Apoptosis is necessary for normal development and removal of transformed cells (White E., 1996; Weil M. et al., 1996,) and plays a central role in pathogenesis of human disease when the genes controlling the apoptotic process are suppressed, overexpressed or altered by mutation (Pitchard U. et al, 1996).

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Both of them converge on the same execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells.

The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. To date, the best characterized ligands and corresponding death receptors include FasL/FasR, and TNF- α /TNFR1. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit

corresponding death domains that bind with the receptors resulting in the auto-catalytic activation of procaspase-8 (Kischkel F. C. et al., 1995).

The intrinsic signaling pathways that initiate apoptosis involve non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens X. et al., 2004). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi which activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" (Chinnaiyan A. M., 1999; Hill M. M. et al., 2004). The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from the mitochondria during apoptosis, translocate to the nucleus and lead to DNA fragmentation (Joza N. et al., 2001; Li L. Y. et al., 2001; Enari M. et al., 1998). The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory S. et al., 2002). To date, a total of 25 genes have been identified in the Bcl-2 family which include anti-apoptotic and pro-apoptotic members. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability.

p53 is considered to be a major player in the apoptotic response to genotoxin. Upon severe damage, p53 is phosphorylated at Ser46 by homeodomain-interacting protein kinase 2 (HIPK2) and subsequently acetylated on Lys382 by the CREB binding protein (CBP). This activation of p53 initiates the expression of its apoptotic target genes such as PUMA, Bax and Apaf-1. p53 also promotes the extrinsic apoptotic pathway by up-regulating Fas receptor and Fas ligand (Petak I. et al., 2000).

These major components of the apoptotic pathway have been well characterized through the use of cell-free biochemical studies and in intact cells using mitotic cell lines. However, recent evidence suggests that apoptosis is regulated very differently between mitotic and post-mitotic cells. Indeed, differently from mitotic cell, the addition of cytochrome c to cytosolic extracts or injection of cytochrome c into the cytosol, is not sufficient to trigger apoptosis in non-cycling cells, such as sympathetic neurons and cardiomyocytes, suggesting that an additional step should be required to induce apoptotic cell death. In post-mitotic cells the X-linked inhibitor of apoptosis protein (XIAP) has been identified as the critical regulator of caspase activation. In sympathetic neurons it has been characterized that the induction of apoptosis relies on both cytochrome c release and XIAP inactivation. In particular, the inhibition of

caspace by XIAP can be overcome by p53-dependent increase in Apaf-1. Consistently, p53 deficient neurons are refractory to relieve the XIAP “brake” and therefore are resistant to DNA damage (Vaughn A.E. et al., 2007). A similar mechanism has been described in myotubes which are resistant to cytosolic cytochrome c microinjection. Importantly, a key role has been attributed to Smac’s release from mitochondria to overcome XIAP and allow cytochrome c to activate caspase and lead to cell death (Smith M.I. et al., 2009).

Moreover, like neurons that are resistant to a variety of stressors, myotubes result radioresistant when compared to myoblasts. The radio-resistance has been molecularly characterized and shown to be due to the uncoupling between ATM activation and p53 phosphorylation, that is required for apoptosis induction (Latella L. et al., 2004).

The restricted apoptosis could be physiologically essential for long term tissue maintenance but it is not clear if this is a general phenomenon in post-mitotic tissues.

4. 2 Autophagy

Autophagy is a physiological mechanism, conserved from yeast to human, which occurs under basal conditions and can be stimulated by stresses such as starvation, various pathologies, or by treatment with pharmacological agents like rapamycin. In addition to its roles in maintaining normal cellular homeostasis by liberating nutrients from macromolecules and by assisting the clearance of misfolded proteins and damaged organelles, autophagy is vital in a range of physiological and pathological situations, including during early embryonic development and neonatal starvation, for the degradation of disease-causing aggregate-prone proteins and in the clearance of pathogenic bacteria. Dysfunction in the autophagy pathway has been implicated in an increasing number of human diseases, from infectious diseases to cancer and neurodegeneration (Mizushima N. et al., 2008).

During autophagy, portions of the cytoplasm, including organelles and proteins, are engulfed by a newly formed membrane, termed a phagophore or isolation membrane, to form a double-membrane vesicle, called autophagosome, which is delivered to lysosomes for hydrolytic degradation (Cecconi F. et al., 2008).

More than 30 different genes regulating autophagy (ATG) have been identified in yeast, and many of these have mammalian orthologs. These genes can be grouped according to their functions at key stages of the autophagy pathway: initiation, elongation, maturation, and fusion with the lysosomes. For example, Beclin 1 is important for localization of autophagic proteins to a pre-autophagosomal structure in the initiation step, whereas the conjugation of the microtubule-associated protein light chain 3 (LC3) with the phospholipids of the vacuolar membrane is required during the elongation stage.

In different cell systems, autophagy is activated by depletion of nutrients or lack of growth factors and, according to current views, this is mediated by the mammalian target of rapamycin (mTOR). Autophagy is suppressed by mTOR, which is in turn controlled directly by the level of intracellular amino acids and indirectly by growth factors *via* Akt/PKB and cell energy status *via* AMPK (Ma X. M. et al., 2009). However, autophagy can also be induced by mTOR-independent mechanisms (Mordier S. et al., 2000).

Emerging evidence indicates that autophagy has an essential role both in cell differentiation and in the preservation of the homeostasis of cells, tissues and organisms (reviewed by Mizushima N. et al., 2010; Mariño G. et al., 2011). Testifying the essential role of autophagy in cell remodelling during erythroid differentiation, mice lacking Ulk1, an autophagic gene, show an increase in the number of reticulocytes and an impaired clearance of both mitochondria and RNA-bound ribosomes (Kundu M. et al., 2008). Moreover, *in vitro* and *in vivo* studies using *Atg5*^{-/-} mice (autophagy defective) had demonstrated the requirement of autophagy during adipocyte differentiation (Baerga R. et al., 2011).

SIRT-1, a nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase protein with a pleiotropic role in the maintenance of homeostasis following genotoxic stress, is one of the major player involved in the crosstalk between autophagy and cell differentiation (Fulco M. et al., 2003; Picard F. et al., 2004; Aymard E. et al., 2011).

If the analysis of several systemic and tissue-specific knockout models of ATG genes have clarified that autophagy plays an important role in mammalian development and differentiation, its involvement in the response to DNA damage is less clear. In particular, whether it functions as a pro-survival or pro-death mechanism upon genotoxic stress is a matter of debate. However, several reports indicate that autophagy can be seen as an integral part of the DDR. A cross-talk between autophagy and a new cytoplasmic function of ATM in response to oxidative stress has been recently described (Alexander A. et al., 2010a; Alexander A. et al., 2010b). Elevated ROS levels induce ATM-dependent-TSC2 activation through the LKB1/AMPK metabolic pathway inhibiting mTORC1 and thus promoting autophagy. This route is p53-independent and ATM is activated by ROS in the cytoplasm suggesting that this pathway is distinct from the canonical activation of ATM in the nucleus upon DNA damage.

An interesting link between DDR and autophagy also emerges from a recent study in yeast (Robert T. et al., 2011). Lack of DDR and DSB repair is observed upon inhibition of histone deacetylase (HDAC) activity, resulting in hyperacetylation of proteins. This is due to decreased association of Sae2 and Exo1 (both involved in DNA end resection during HR) with DSB ends and decreased levels of Sae2 protein. Interestingly, inhibition of mTOR activity and thus triggering of autophagy also result in decreased levels of Sae2. This model suggests that severely damaged DNA and associated machinery are removed

from the nucleus *via* an autophagic process regulated by the acetylation status of key repair proteins. This may be a mechanism for keeping DNA repair enzymes away from cellular DNA that is not damaged, avoiding accidental "repair" of replicating DNA.

Although the role of autophagy in the control of post-mitotic tissue integrity is broadly accepted, its involvement in the maintenance of genome stability during cell differentiation is still not elucidated.

In this study, the characterization of the response to DNA damage and the analysis of basal autophagy during muscle differentiation have been performed in order to investigate a possible involvement of the autophagic pathway in the dealing with genotoxic insults.

II. RESULTS

DNA SSBs accumulate in myotubes but do not lead to cell death

DNA repair capacity in post mitotic cells has been exploited by the use of a skeletal muscle cell differentiation *in vitro* system in which actively proliferating myoblasts are induced to differentiate in myotubes under appropriate cell culture conditions. A clear decrease in BER capacity of terminally differentiated muscle cells was observed and it was ascribed to the lack of DNA ligase I and the downregulation of XRCC1 affecting both the long- and the short- patch of BER. Consistently, myotubes accumulate DNA single-strand breaks (SSB) upon oxidative stress (Narciso L. et al., 2007).

We carried out a further characterization of BER, focusing our analysis on the first enzymatic step of this pathway, which implies the removal of the damaged base by specific DNA glycosylases giving rise to apurinic/apyrimidinic sites rapidly converted into SSB by the action of AP endonucleases and lyases.

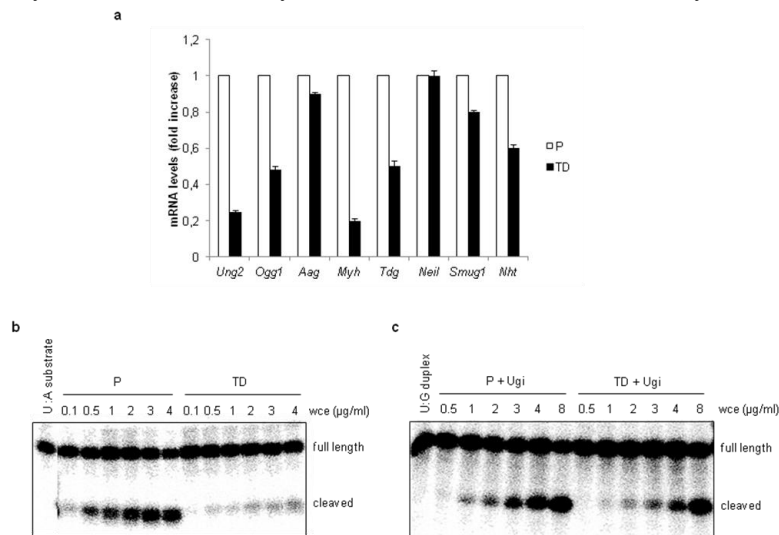


Figure 6. DNA glycosylases are downregulated in terminally differentiated muscle cells. (a) Gene expression analysis of several DNA glycosylases by RT-PCR in proliferating (P) and terminally differentiated (TD) cells. (b) UNG2 activity was measured by an *in vitro* functional assay. An 32 P-end-labeled duplex oligonucleotide (30 mer) which contains a single U:A mismatch was incubated with different concentrations of whole cell extracts (wce) from proliferating (P) and terminally differentiated (TD) cells. (c) SMUG1 activity was measured by an *in vitro* functional assay. For this activity a 32 P-end-labeled duplex oligonucleotide (30 mer) containing a single U:G mismatch was used as substrate and incubated at different concentrations of whole cell extracts (wce) from proliferating (P) and terminally differentiated (TD) cells. For the SMUG 1 activity assay Ugi, a specific inhibitor of UNG2, was added to the reactions. Full length and cleaved products were analysed by polyacrylamide denaturing gel electrophoresis and quantified by electronic autoradiography (Instant Imager). Error bars indicate S.D.

We measured the gene expression levels of different DNA glycosylases and found that *Ung2* (uracil-DNA glycosylase2) and *Myh* (human mutY homolog) were strongly downregulated in post-mitotic muscle cells compared to the proliferating counterpart (Fig. 6a). This was in agreement with the involvement of these two enzymes in the removal of lesions formed during S-phase. The activity of UNG2, responsible for uracil removal from U:A mismatch, was also impaired in myotubes as demonstrated by a functional assay with a specific substrate (Fig. 6b), further confirming a clear down-regulation of this cell cycle-associated glycosylase in terminally differentiated cells. The presence of uracil in the genome can be due not only to replication errors, but also to spontaneous deamination of cytosine leading to U:G mismatch. The importance of ensuring uracil removal is testified by the maintenance of functionality of SMUG1 (an uracil DNA glycosylase which removes uracil from ssDNA during transcription) in non-cycling cells such as myotubes (Fig. 6c). In agreement with BER impairment in terminally differentiated cells, the exposure to methyl methanesulfonate (MMS), an alkylating agent that induces SSB as intermediates during the BER process, led to DNA lesions accumulation (Fig. 7a) which was mirrored by phosphorylation of H2AX (Fig. 7b).

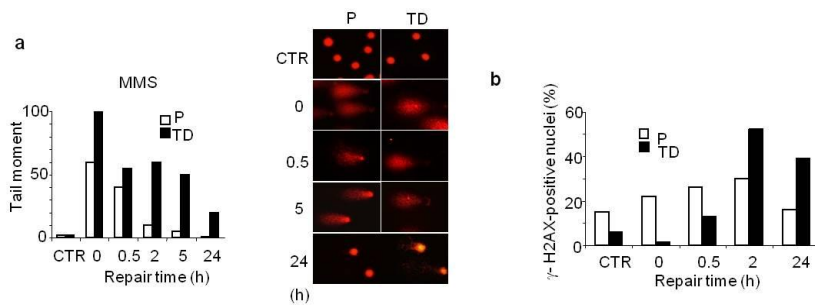


Figure 7. Terminally differentiated muscle cells accumulate DNA SSB after MMS exposure. (a) Left: Myoblasts (P) and myotubes (TD) were exposed to MMS (1mM, 30 min) and DNA SSB were analyzed by the comet assay at different repair times. The average of the tail moment, calculated by multiplying the total intensity of the comet tail by the migration distance from the centre of the comet head, was used as measure of DNA damage. At least 100 cells per experimental point is shown. Right: Microphotographs of proliferating (P) and terminally differentiated (TD) cells subjected to the comet assay and stained with ethidium bromide. Representative untreated control cells (CTR) and after different post-treatment times are shown. Experiments were repeated twice and one representative experiment is shown. (b) DNA damage induction and repair as detected by γ H2AX foci formation after exposure to MMS (3mM, 30 min) in proliferating (P) and terminally differentiated (TD) cells.

These data strongly suggest that in postmitotic cells which are definitely impaired in replication ability, the phosphorylation of H2AX is induced by BER

intermediates per se without any replication-dependent turning into DSB. To better understand the fate of persistent SSB in the genome of post-mitotic muscle cells, proliferating and terminally differentiated cells were exposed to different DNA damaging agents, such as MMS and camptothecin (CPT), a Topoisomerase1 (TOP1)-linked SSB inducer (Pommier Y. et al., 2010). Cell survival was measured by counting metabolically active cells. At doses that killed over 50% of the myoblasts, myotubes were resistant to the toxic effects of DNA SSB (Fig. 8).

Myotubes were also resistant to H₂O₂ (data non shown) but the comparison with myoblasts was hampered by the induction of differentiation upon H₂O₂ exposure (Kuster G. M. et al., 2010). Terminally differentiated muscle cells are therefore not only resistant to IR (Fig. 8; Latella L. et al., 2004) but also to SSB-inducing agents.

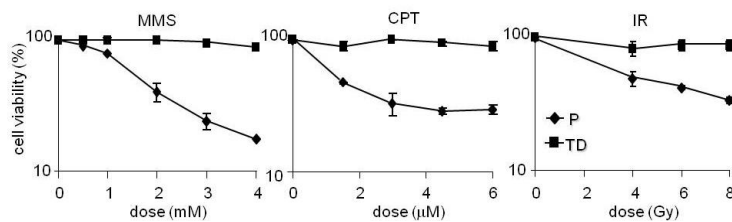


Figure 8. Myotubes are resistant to the toxic effects of SSB inducers and IR. Proliferating (P) and terminally differentiated (TD) cells were exposed to different doses of MMS, CPT (30 min treatment and 3 h respectively) and IR and cell survival was measured by counting metabolically active cells as measured by CCK-8 72 h after treatment. Error bars indicate S.D.

γ-H2AX response is activated in myotubes upon SSB induction and ATM is the main kinase involved

Oxidative (Narciso L. et al., 2007) and alkylation (Fig. 7b) damage is able to activate the γH2AX response in post-mitotic muscle cells. H2AX can be phosphorylated by ATM, DNA-dependent protein kinase (DNA-PK) or ATR. In myotubes ATR is missing but either ATM or DNA-PK could play this role. To identify the candidate kinase, myotubes were pre-treated with either an ATM (KU55933) or a DNA-PK kinase (NU7441) inhibitor and the induction of phosphorylated H2AX was monitored in response to H₂O₂ or MMS by both immunofluorescence and western blotting analysis (Fig. 9).

Interestingly, whereas the H₂O₂-induced H2AX phosphorylation was fully suppressed by the ATM inhibitor, it was not affected by NU7441 (Fig. 9a). Notably, when this strategy was used after exposure to MMS, ATM resulted to

have a role in the activation of γ H2AX response, but with a different kinetics. This kinase was only involved in the H2AX phosphorylation events arising at later times after damage suggesting that only a subset of alkylation-induced lesions were able to trigger ATM-dependent damage signalling. The DNA-PK inhibitor was ineffective on MMS-induced γ H2AX response too (Fig. 9b, left).

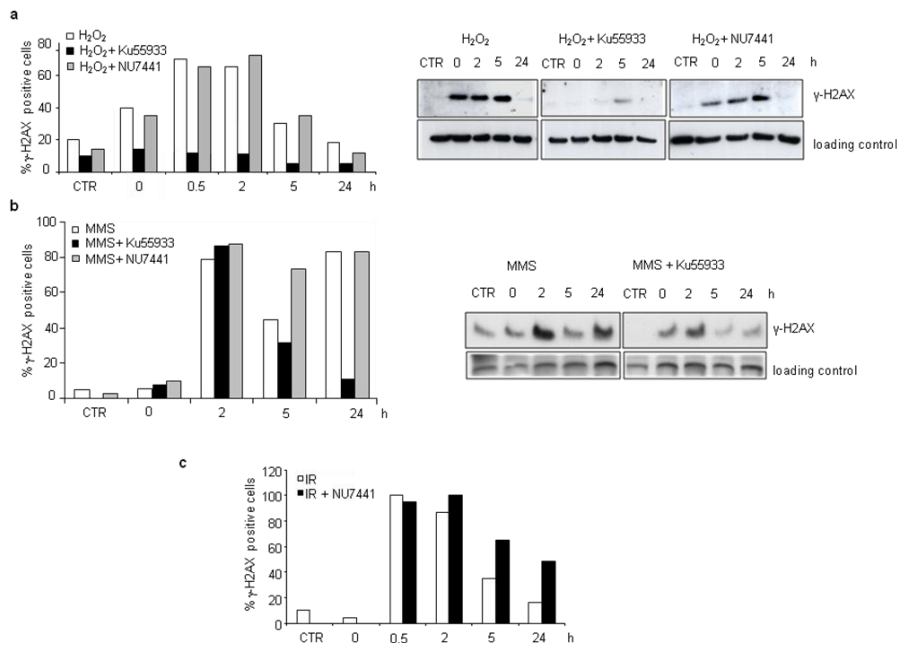


Figure 9. γ H2AX response is activated in myotubes upon SSB induction and ATM is the main kinase involved. Induction of γ H2AX foci after H₂O₂ (a), MMS (b) or IR (c) treatment of terminally differentiated muscle cells. Myotubes were exposed to the DNA-damaging agent with or without 1 h pre-treatment with a specific kinase inhibitor (KU55933 or NU7441) and analyzed at different times after damage. (a) DNA damage induction and repair as detected by γ H2AX foci formation (left) or western blotting (right) after exposure to H₂O₂ (100 μ M, 30 min). (b) DNA damage induction and repair as detected by γ H2AX foci formation (left) or western blotting (right) after exposure to MMS (3mM, 30 min). (c) DNA damage induction and repair as detected by γ H2AX foci formation after exposure to IR (2 Gy). Experiments were repeated twice and one representative experiment is shown. In the γ H2AX foci assay at least 200 nuclei were examined for each time point. Western blot analysis was performed on nuclear cell extracts and the loading control is DNA polymerase β . CTR: untreated control cells.

If on one hand DNA-PK inhibition did not prevent the reversal of H2AX phosphorylation upon exposure to H₂O₂ or MMS (Fig. 9a), on the other hand it significantly affected H2AX dephosphorylation following exposure of myotubes to IR (Fig. 9c). Therefore DNA-PK-dependent non homologous end-joining

(NHEJ) is fully operational in myotubes but pathways other than NHEJ should be involved in the repair of H₂O₂-induced lesions.

These results provide a clear evidence that activation of ATM is involved in SSB signalling depending on the type of damage. γ H2AX activation initiates SSB processing *via* a pathway that is DNA-PK independent.

The expression of genes involved in DDR and apoptosis is modulated during differentiation

The ability of myotubes to tolerate DNA damage prompted us to investigate the status of DDR genes of these post-mitotic cells. It is known that differentiation involves significant gene reprogramming including genes involved in DDR and apoptosis (Fisher C. L. et al., 2011). We observed that p53 was still expressed at protein level during early time of differentiation but it strongly declined in cells undergoing terminal differentiation (Fig. 10a).

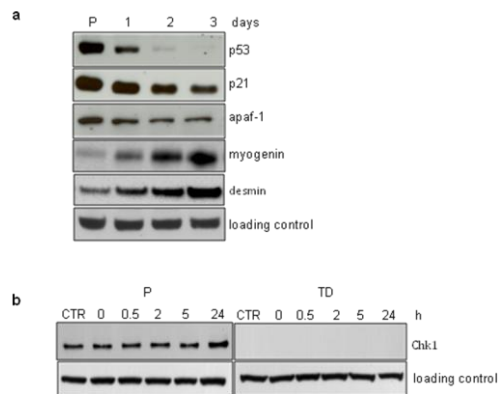


Figure 10. The expression of DDR and apoptosis genes changes during differentiation. (a) Immunoblotting analysis on whole extracts from proliferating cells (P) and at different differentiation days. Loading control: human AP endonuclease 1 (HAP1). (b) Protein levels of Chk1 in proliferating (P) and terminally differentiated (TD) cells either untreated (control, CTR) or at different times after exposure to H₂O₂ (100 μM, 30 min). Loading control: β-tubulin.

Similarly, high levels of p21 seemed to be required to trigger differentiation (at day 1) but then a decrease was observed as a function of the differentiation time. It is important to note that although the levels of this cyclin-dependent kinase inhibitor decrease, it is required for maintaining the post-mitotic state of myotubes (Pajalunga D. et al., 2007). The apoptosis protease-activating factor

Apaf-1 was progressively downregulated during muscle cells differentiation. The decrease in Apaf-1 of myotubes has been associated with their resistance to apoptosis (Smith M. I. et al., 2009). As expected, myogenin and desmin, markers of skeletal myogenesis, increased during differentiation.

Downregulation and functional inactivation of genes implicated in cell cycle checkpoint signalling, such as Chk1 and ATR is a feature of myotubes (Lukas C. et al., 2001; Puri P. L. et al., 2001). As shown in Fig. 10b, myoblasts expressed Chk1 both when they were untreated or exposed to hydrogen peroxide whereas in post-mitotic cells Chk1 was missing also after DNA damage. Conversely, ATM and Chk2 are maintained in differentiating cells also after damage (Lukas C. et al., 2001; Latella L. et al., 2004) testifying that the ATM/Chk2 module is an active kinase branch in myotubes.

The p53 downstream signalling is impaired in myotubes upon SSB induction

To further investigate the signalling cascade after SSB induction, the p53 gene response was investigated in proliferating and terminally differentiated cells after exposure to SSB-inducing agents. The levels of total p53 and its serine (Ser) 18 (human Ser 15) phosphorylated form (pp53) were monitored after exposure to H₂O₂, MMS and CPT (Fig. 11).

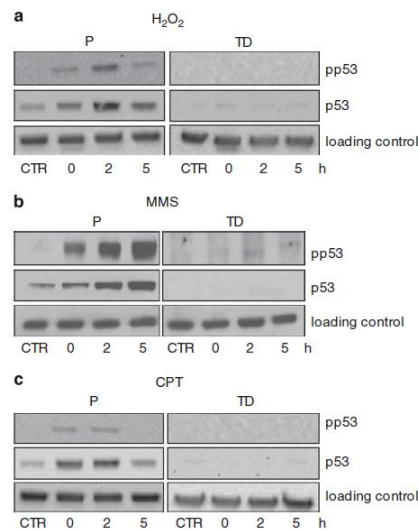


Figure 11. The p53 response is impaired in myotubes after exposure to SSB inducers. The levels of p53 and phosphorylated p53 (pp53) were monitored in proliferating (P) and terminally differentiated (TD) cells at different times after exposure to (a) H₂O₂ (100 μM, 30 min), (b) MMS (3 mM, 30 min) and (c) CPT (6 μM, 3 h). Western blot analysis was conducted on whole cell extracts. Loading control: human AP endonuclease 1 (HAP1).

In proliferating cells p53 stabilization and phosphorylation invariably increased after SSB induction, albeit with different kinetics, in sharp contrast to what observed in differentiated cells. Thus, the defective p53 response and protection from death of myotubes in response to DNA break-inducing agents might be due to the inability to stabilize p53 or to prevention of transcription of some p53 target genes. To distinguish between these two possibilities we used nutlin-3 that, by inhibiting the interaction between p53 and MDM2, stabilizes p53 and activates the p53-dependent cell death pathway (Shangary S. et al., 2009). As shown in Fig. 12, nutlin-3 was able to stabilize p53 in untreated as well as MMS-treated myotubes without inducing toxicity (Fig. 12b).

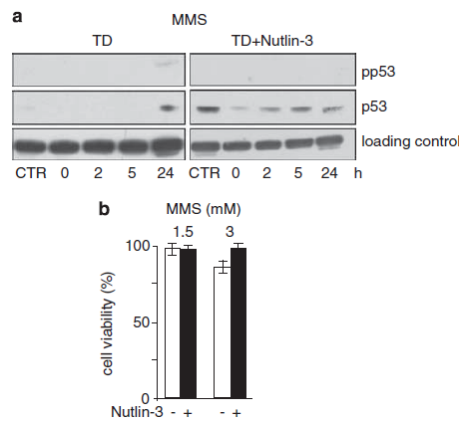


Figure 12. Nutlin 3-dependent p53 stabilization does not sensitize the myotubes to the cytotoxic effect of MMS. (a) Western blot analysis of p53 and phosphorylated p53 (pp53) in whole extracts of terminally differentiated (TD) cells at different times after MMS exposure (3mM, 30 min). Cells were also pre-incubated with nutlin-3 (1 μ M) before MMS exposure. Loading control: GAPDH. CTR: untreated control cells. (b) Cell survival was measured in myotubes following exposure to 1.5 and 3 mM MMS alone or after incubation with nutlin-3. Metabolically active cells were counted by the CCK-8 assay 48 h after treatment. Error bars indicate S.D.

The levels of p53 did not change during the additional 24 h that is the maximal recovery time after damage used in our experiments. As expected nutlin-3 did not affect the phosphorylation of p53.

It is of interest to note that as a consequence of p53 stabilization during differentiation Apaf-1 mRNA levels are not decreased (Fig. 13) but this is not sufficient to trigger cell death when myotubes are exposed to MMS.

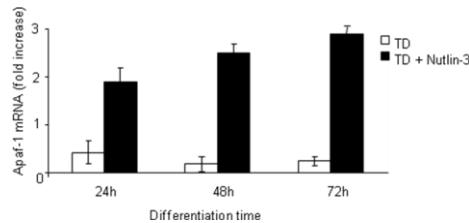


Figure 13. Apaf-1 is not downregulated during differentiation if p53 is stabilized. Analysis of mRNA levels of Apaf-1 by real time-PCR in myotubes at 24, 48 and 72 h of differentiation with (black squares) or without (white squares) nutlin-3 (1 μ M, 72h).

We can conclude that upon DNA break induction there is an uncoupling between ATM activation and p53 downstream signalling that prevents the activation of the cell death pathway.

The p53 cell death pathway is activated in myotubes by doxorubicin and menadione

Although myotubes are resistant to SSB-inducers and IR, the p53 cell death pathway is still functional. As previously shown (Latella L. et al., 2004), myotubes are extremely sensitive to the anthracycline derivative doxorubicin (DOXO) (Fig. 14a). The p53 response was activated in myotubes upon DOXO treatment, as testified by the increase of total p53 and its phosphorylated form as a function of the post-treatment time (Fig. 14b). As expected DOXO-induced cell death was exacerbated ($p < 0.05$) in the presence of nutlin-3 that further stabilized p53 (Fig. 14c). DOXO induces a variety of DNA lesions, including DSB arising by Topoisomerase2 (TOP2) inhibition and oxidative damage (Pommier Y. et al., 2010). All of them might concur to the activation of the p53-dependent cell death pathway. Several studies suggest that the toxicity exerted by TOP2 inhibitors is due to proteasomal processing of TOP2-DNA adducts that exposes TOP2-concealed DSB (Zhang A. et al., 2006; Lyu Y. L. et al., 2007). In agreement with this model the proteasome inhibitor MG132 attenuated ($p < 0.05$) DOXO-induced lethality in myotubes (Fig. 14d). Conversely, the ROS-scavenger N-acetylcysteine (NAC) did not affect the cytotoxicity induced by DOXO (Fig. 14d) suggesting that in muscle cells the redox cycling ability of DOXO is not the main cause of toxicity. These results are consistent with the resistance to the toxic effect induced by hydrogen peroxide which we previously observed in myotubes.

Since mitochondrial integrity is functional to maintain homeostasis of muscle tissue, we investigated the response of terminally differentiated muscle cells to

the toxic effects of the mitochondria-specific inhibitor menadione (MND) (Loor G. et al., 2010).

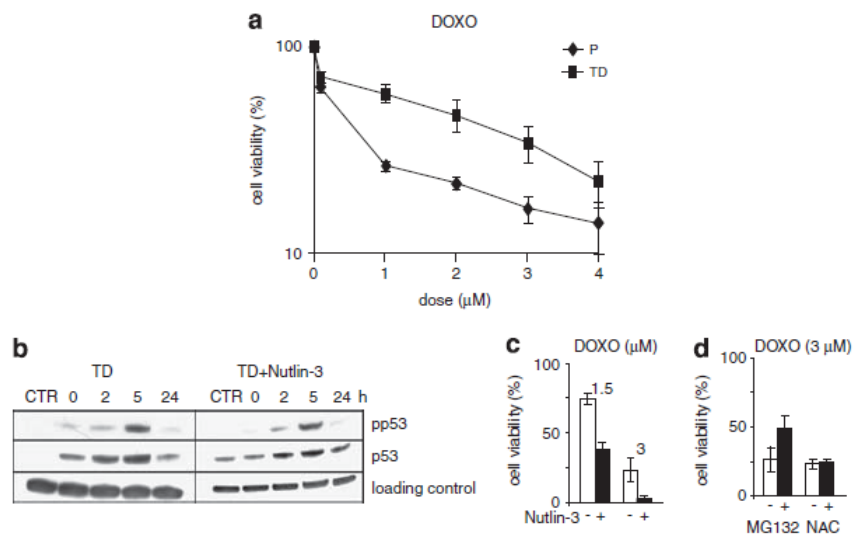


Figure 14. The p53 cell death pathway is activated in myotubes exposed to doxorubicin. (a) Cell survival was measured in proliferating (P) and terminally differentiated (TD) cells following exposure to various doses of DOXO for 3 h. Metabolically active cells were counted by the CCK-8 assay 48 hours after treatment. Error bars indicate S.D. (b) Western blot analysis of p53 and phosphorylated p53 (pp53) in whole extracts of myotubes at different times after DOXO exposure (3 μM; 3 h) alone or with nutlin-3 (1 μM) pre-incubation. Loading control: GAPDH. CTR: untreated control cells. (c) Cell survival was measured in myotubes following exposure to 1.5 and 3 μM DOXO with or without nutlin-3. Metabolically active cells were counted by the CCK-8 assay 48 h after exposure. Error bars indicate S.D. (d) Cell survival was measured in myotubes following exposure to 3 μM DOXO with or without pre-incubation with MG132 (0.5 μM for 30 min) or NAC (0.5 mM for 30 min). Error bars indicate S.D.

As shown in Fig. 15a MND induced a dramatic decrease of myotube vitality comparable to that of their proliferating counterpart. In sharp contrast to the effects of H₂O₂-induced nuclear ROS, after exposure to MND p53 was stabilised and activated by phosphorylation (Fig. 15b) suggesting a crucial role of oxidative stress at mitochondrial level in myotubes integrity. Consistently, preincubation of myotubes with the anti-oxidant NAC almost abolished ($p < 0.05$) the cytotoxic effects of MND. Notably, proteasome inhibition by MG132 significantly increased the MND-induced toxicity ($p < 0.05$) which is likely due to the prevention of p53 degradation (Fig. 15c). All together these experiments indicate that post-mitotic muscle cells are protected by DNA damage induced cell death by defective signalling downstream of p53 but this blockade is overcome when specific cellular target are damaged.

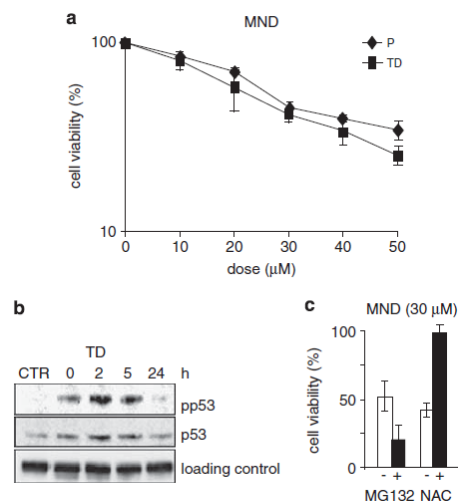


Figure 15. The p53 cell death pathway is activated in myotubes exposed to menadione. (a) Cell survival was measured in proliferating (P) and terminally differentiated (TD) cells following exposure to various doses of MND for 1 h. Metabolically active cells were counted by the CCK-8 assay 48 h after treatment. Error bars indicate S.D. (b) Western blot analysis of p53 and phosphorylated p53 (pp53) in whole extracts of myotubes at different times after MND exposure (20 µM for 1 h). Loading control: GAPDH. CTR: untreated control cells (c) Cell survival was measured in myotubes following exposure to 30 µM MND with or without pre-incubation with MG132 (0.5 µM for 30 min) or NAC (0.5 mM for 30 min). Metabolically active cells were counted by the CCK-8 assay 48h after treatment. Error bars indicate S.D.

p53 activation is required for the execution of cell death in myotubes

To further investigate the role of p53 in the execution of cell fate decisions, satellite cells were derived from p53 null mice, induced to differentiate and then treated with MMS, DOXO or MND. A significant protection from cell killing was recorded after exposure to DOXO and MND whereas, as expected, no modulation was observed after MMS treatment (Fig. 16a).

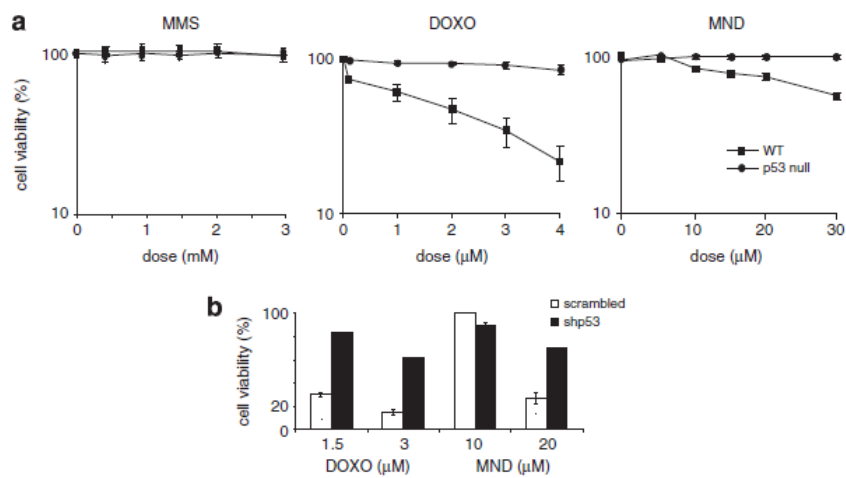


Figure 16. p53 is required for the execution of cell death in myotubes, but the repertoire of genes activated is restricted. (a) Wild-type and p53-null myotubes were exposed to various doses of MMS, DOXO or MND, and cell survival was measured by counting the metabolically active cells by the CCK-8 assay 48 h after treatment. Error bars indicate S.D. (b) Stable silenced p53 myotubes (transfected with a vector containing a p53 shRNA) and control myotubes (transfected with a vector containing a scrambled sequence) were exposed to DOXO and MND at the indicated doses, and cell survival was measured by counting the metabolically active cells 48 h after treatment. Error bars indicate S.D.

Although muscle cells lacking p53 have a reduced ability to differentiate (Loo G. et al., 2010), under our experimental conditions we were able to achieve a differentiation efficiency above 80% thus limiting the possible effects of contamination by proliferating cells. In order to confirm these results, the experiments were conducted in p53-downregulated satellite cells obtained by short hairpin RNA (shRNA) interfering (Fig. 16b). A clear protection ($p < 0.05$) from cytotoxicity was observed after exposure to DOXO and MND. Interestingly, despite the activation of cell death by DOXO and MND is fully p53-dependent, terminally differentiated cells had a restricted repertoire of genes activated by p53. The p53 target genes, p21 and Bax, was not

transactivated following exposure to either DOXO or MND (Fig. 17) whereas a clear p21 transactivation was observed in myoblasts after DNA damage (data not shown).

These results led us to investigate which is the cell death pathway induced in post-mitotic muscle cells.

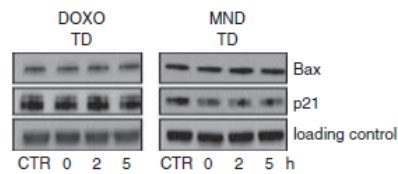


Figure 17. The repertoire of activated p53 target genes is restricted. Western blot analysis of Bax and p21 in whole extracts from myotubes exposed to DOXO (3 mM for 3 h) or MND (30 mM MND for 1 h). CTR, untreated control cells. Loading control, GAPDH.

Characterization of autophagic pathways during differentiation

Recent lines of evidence indicate that autophagy is important for cell differentiation but its role in response to DNA damage is less clear (see section 4.2). In order to investigate the contribution of autophagy in cell death or survival of myotubes upon genotoxic stress, we first analysed the basal level of autophagy during the differentiation of myoblasts into myotubes. The conversion of LC3 I into the lipidated form LC3 II, present both on the inner and the outer membrane of the autophagosomes, is a well known marker of autophagy induction. However, an increase in LC3 II levels may represent either the increased generation of autophagosomes and/or a block in autophagosomal maturation and the completion of the autophagy pathway. In order to distinguish between these two possibilities we used a combination of Leupeptin (LEU) and ammonium chloride (NH₄Cl) which inhibit the lysosomal proteases and the acidification of the lysosomes leading to LC3 II accumulation respectively. Myoblasts were induced to differentiate with or without LEU/NH₄Cl and harvested at different times (24, 48 and 72 h). Protein analysis has carried out by western blot. A mild increase in LC3 II levels was detected at 72 h after differentiation induction (Fig. 18a). In the presence of LEU/NH₄Cl a clear accumulation of LC3 II was already observed at 24 h and a further increase was detectable at 72 h suggesting that during muscle differentiation the autophagic flux is so rapid that LC3 II accumulates only preventing its degradation.

Since it is known that mTOR is the negative regulator of autophagy, we performed a western blot analysis to check the phosphorylation status of one of its target, S6 ribosomal protein, during differentiation. Surprisingly, we found that despite autophagy induction, mTOR was not inactivated as shown by

phosphorylation of its target (Fig. 18b). The induction of autophagy during muscle differentiation was confirmed by following colocalization of LC3 and lysosome-associated membrane protein (LAMP) by immunofluorescence analysis (Fig. 18c). Recent studies have revealed that specific substrates are preferentially degraded by autophagy and p62 is one of the best studied example. p62 is selectively incorporated into autophagosomes through direct binding to LC3 and it is efficiently degraded by autophagy (Bjørkøy et al., 2005). Consequently, the total cellular levels of p62 is inversely correlated with autophagic activity.

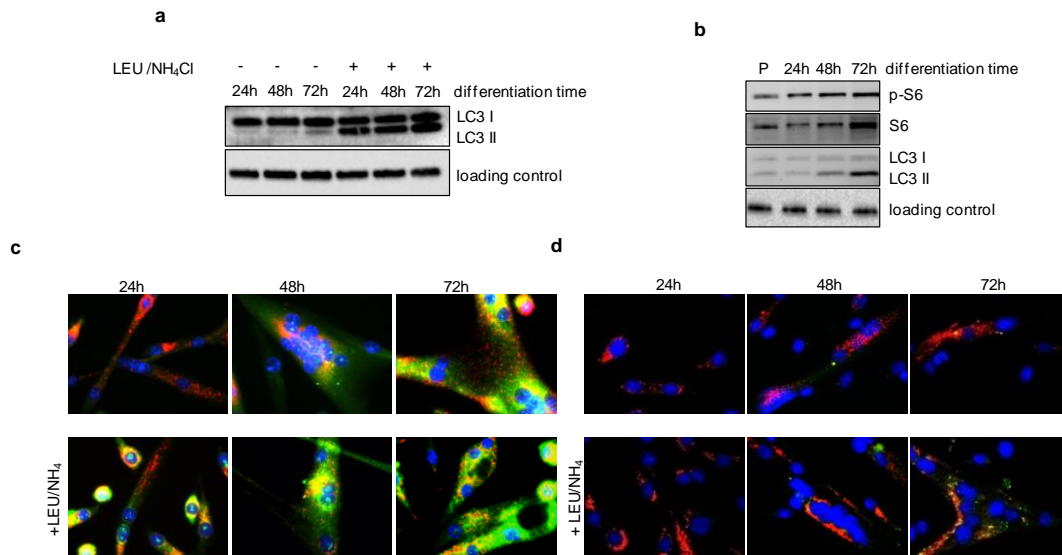


Figure 18. Autophagy is induced during muscle cells differentiation and is independent by mTOR inactivation. (a) Western blot analysis of whole cell extracts of myoblast induced to differentiate and collected after 24, 48 and 72 h in the presence or absence of LEU/NH₄Cl (100 μM/20 mM, 2h). (b) Western blot analysis of phosphorylated S6 (pS6), S6, LC3 I and LC3 II in whole cell extracts of myoblast (P) and myotubes (24, 48 and 72 h). Loading control: p38MAPK. (c) Immunofluorescence analysis of myotubes at 24, 48, and 72 h of differentiation with or without LEU/NH₄Cl (100 μM/20 mM, 2h). Lysosome-associated membrane protein (LAMP) (red) and LC3 staining (green). (d) Myotubes at 24, 48, and 72 h of differentiation with or without LEU/NH₄Cl (100 μM/20 mM, 2h) were immunostained with LAMP (red) and p62 (green). Nuclei were counterstained with DAPI. At least 200 nuclei were examined for each time point. Experiments were repeated thrice and one representative experiment is shown.

In addition, the immunofluorescence analysis showed that during the differentiation process p62 was not detectable but only accumulated in the presence of LEU/NH₄Cl testifying that during muscle differentiation the

autophagic flux is active (Fig.18d). Altogether these data suggest that during the *in vitro* differentiation of myotubes, autophagy is induced and is independent of mTOR inactivation, in agreement with previous findings in C2C12 cells (Tanida I. et al., 2006).

In order to further characterize the autophagic pathway, myoblasts were induced to differentiate with or without rapamycin, a well known mTOR inhibitor. The mTOR activation, testified by inhibition of S6 phosphorylation (Fig. 19a) does not modulate muscle differentiation extension as testified by myosin heavy chain (MHC) and desmin induction.

Rapamycin increased autophagy induction as shown by the increase in LC3 II levels in absence and in presence of LEU/NH₄Cl (Fig. 19b and c) and by the decrease of p62 (Fig. 19b). Thus, the mTOR inactivation-dependent autophagic pathway is functional in muscle cells although is not the pathway underlying the basal autophagy and is not involved in differentiation.

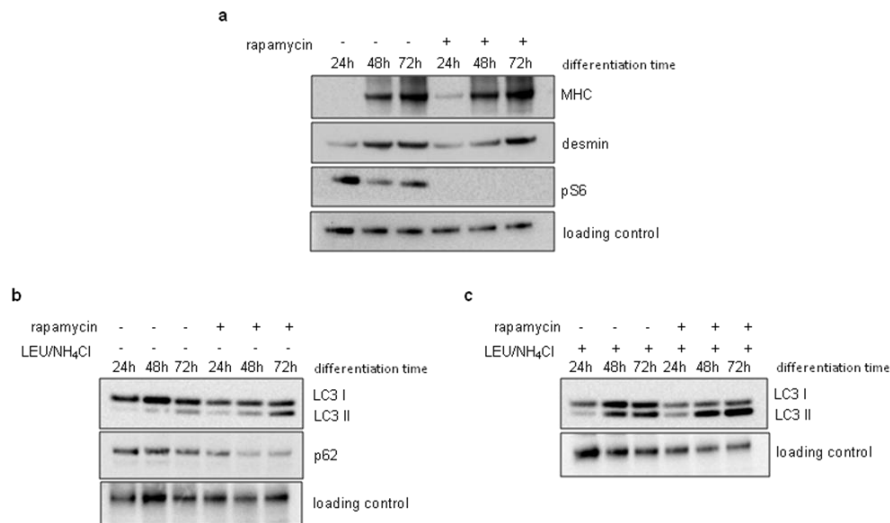


Figure 19. (a) Western blot analysis of myosin heavy chain (MHC), and phosphorylated S6 protein in whole cell extracts of myotubes at 24, 48 and 72 h of differentiation with or without 20 nM rapamycin treatment (every 24 h). (b) Western blot analysis of LC3 I, LC3 II and p62 in whole cell extracts of myotubes (24, 48 and 72 h of differentiation) treated with rapamycin (20 nM, renewing it every 24 h) alone and (c) with LEU/NH₄Cl (100 μM/20 mM, 2h). Loading control: p38MAPK.

The role of p53 in regulation of autophagy

It was described by Tasdemir E. and coworkers that degradation of p53 is necessary to activate autophagy (Tasdemir E. et al., 2008). During differentiation a significant gene reprogramming occurs and involves p53 downregulation in myotubes compared to myoblasts. To verify the possibility that a reduction in p53 levels was required for autophagy onset, myoblasts were induced to differentiate with or without nutlin-3, a p53 stabiliser, and analysed during differentiation time. p53 stabilization did not affect either the differentiation process or the induction of autophagy (Fig. 20).

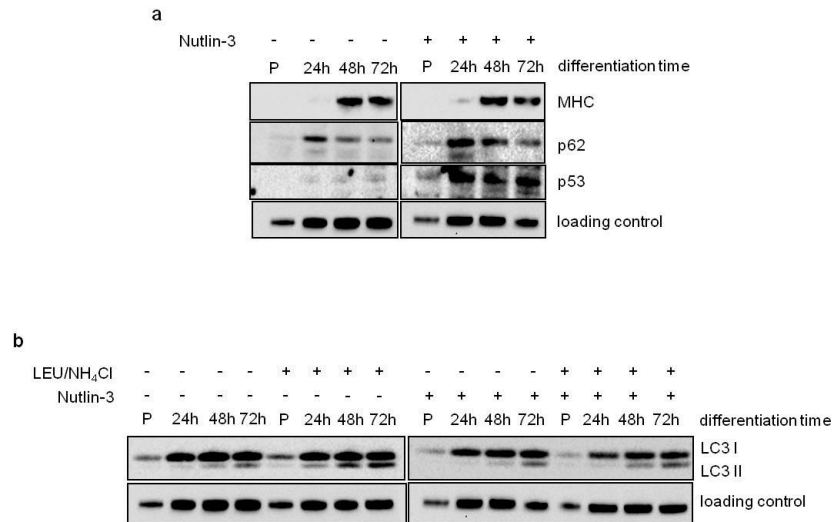


Figure 20. p53 stabilization by Nutlin- 3 does not affect autophagy during differentiation.

(a) Western blot analysis of myosin heavy chain (MHC), p62 and p53 in whole cell extracts of myoblasts (P) and myotubes (24, 48 and 72 h of differentiation) treated or not with 1 μ M Nutlin-3. (b) Western blot analysis of LC3 I and LC3 II in whole cell extracts of myoblasts (P) and myotubes (24, 48 and 72 h of differentiation) treated or not with 1 μ M Nutlin-3 and with or without LEU/NH₄Cl (100 μ M/20 mM, 2h). Loading control: p38MAPK.

We can also exclude a delay in the autophagic flux since degradation of p62 occurred both in the presence and in the absence of nutlin-3 (Fig. 20a). Collectively, these data suggest that p53 is not involved in the induction of autophagy during muscle differentiation.

Analysis were also carried out during differentiation of p53 null muscle cells. The expression of the differentiation marker MHC as well as the LC3 II levels during differentiation time were comparable to what observed in the wild type cells (Fig. 21). Similarly to what observed in p53 proficient cells, in the presence of LEU/NH₄Cl during differentiation both LC3 II accumulation (Fig. 21a) and p62 levels reduction (Fig. 21b) were observed.

Moreover, the analysis of the S6 phosphorylation status showed that even in a p53 null background the basal autophagic response which flanks muscle differentiation process follows a mTOR inactivation independent pathway (Fig 21b).

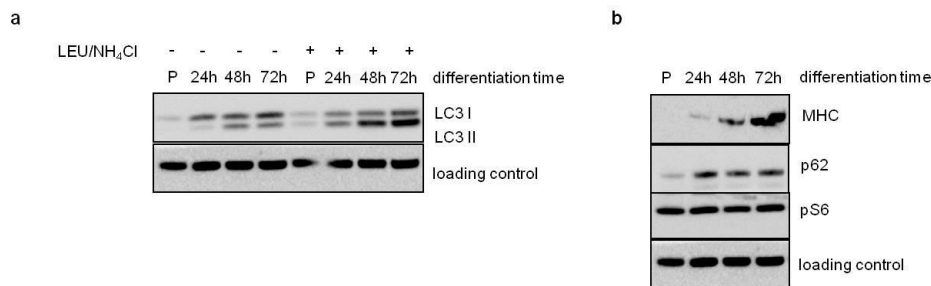


Figure 21. In the absence of p53 autophagy is maintained. (a) Western blot analysis of LC3 I and LC3 II in whole cell extracts of p53 null myoblasts (P) and myotubes (24, 48 and 72 h of differentiation) with or without LEU/NH₄Cl treatment (100 μ M/20 mM, 2h). (b) Western blot analysis of myosin heavy chain (MHC), p62 and phosphorylated S6 protein (pS6) in whole cell extracts of p53 null myoblasts (P) and myotubes (24, 48 and 72 h of differentiation). Loading control: p38MAPK.

The cross-talk between autophagy and differentiation

Since we found that autophagy was induced during muscle differentiation, we wanted to investigate whether it is essential to the myogenic program. In order to address this question we downregulated *Beclin 1*, involved in the localization of autophagic proteins to a pre-autophagosomal structure, by small interfering RNA (siRNA) and analysed the protein level of specific muscle differentiation and autophagic markers during differentiation time. Interestingly, the downregulation of *Beclin 1* modulated both differentiation and autophagy as testified by the reduction of MHC (Fig. 22a and c) and LC3II levels in silenced cells versus the native counterpart. As expected, the lysosomal proteases

inhibition leads to the accumulation of LC3 II without any modulation of MHC protein levels (Fig. 22a).

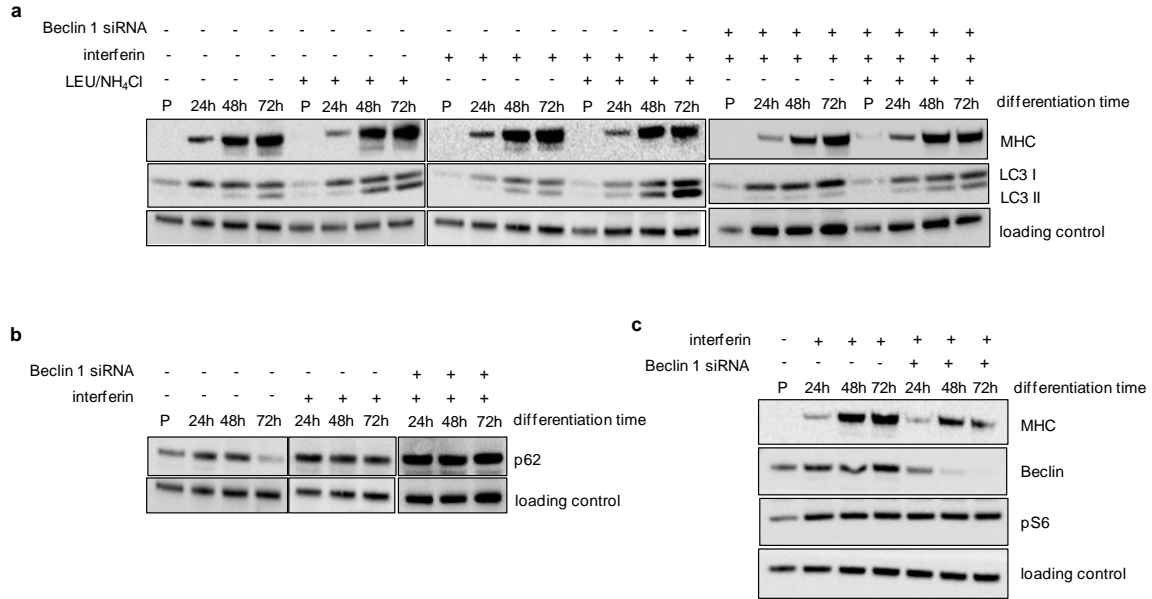


Figure 22. The autophagic flux is delayed by silencing of Beclin 1. (a) Western blot analysis of myosin heavy chain (MHC), LC3 I and LC3 II in whole cell extracts of myoblasts (P) and myotubes (24 h, 48 h and 72 h of differentiation) treated with 20nM Beclin 1 siRNA or interferin and LEU/NH₄Cl (100 μ M/20 mM, 2h). (b) Western blot analysis of p62 in whole cell extracts of myoblasts (P) and myotubes (24 h, 48 h and 72 h of differentiation) treated with 20nM Beclin 1 siRNA or interferin. (c) western blot analysis of myosin heavy chain (MHC), Beclin 1 and phosphorylated S6 protein (pS6) in whole cell extracts of myoblasts (P) and myotubes (24 h, 48 h and 72 h of differentiation) with or without Beclin 1 siRNA or interferin. Loading control: p38MAPK.

Moreover, *Beclin 1* siRNA stabilised p62 levels indicating a block in the autophagic flux (Fig. 22b). Although the autophagy impairment observed in *Beclin 1*-silenced cells, the phosphorylation of the S6 protein still testified the mTOR activation (Fig. 22c).

Immunofluorescence analysis showed that the efficiency of differentiation, (nuclei belonging to MHC positive cells/total nuclei), was not influenced by *Beclin 1* silencing whereas the fusion ability (nuclei inside myotubes/number of myotubes) of myocytes in multinucleated syncytia was clearly reduced (Fig.

23b) suggesting that autophagy could be involved in this step during muscle differentiation process.

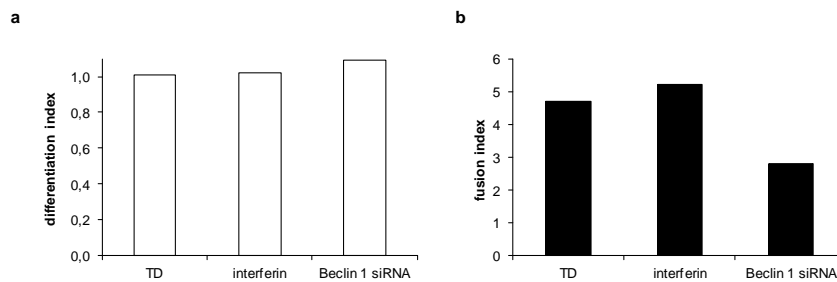


Figure 23. Beclin 1 silencing affects multinucleated myotubes formation Immunofluorescence staining for myosin heavy chain (MHC) of myotubes at 72 h of differentiation (TD) and in presence of interferin or Beclin 1 siRNA. (a) Differentiation index was calculated as ratio of nuclei belonging to MHC positive cells on total nuclei. (b) Fusion index was calculated as ratio of nuclei belonging to MHC positive cells possessing three or more nuclei on total MHC positive cells. At least 200 nuclei were examined for each time point. Experiments were repeated twice and one representative experiment is shown.

p53 and metabolism during muscle differentiation

Cell metabolism is strongly regulated by p53. Several studies have shown that p53 has a role in the regulation of glycolysis that results to be inhibited by p53 at various steps (Vousden K. H. et al., 2009). To investigate whether the physiological downregulation of p53 observed during muscle differentiation was able to affect the metabolism of myotubes compared to myoblasts, a metabolic profile of skeletal muscle cells was analysed during differentiation (Green et al., 2006). A comparative quantification of aqueous metabolites by ¹H-NMR spectroscopy showed that in p53 null cells the levels of lactate were higher than in wild type cells (Fig. 24a and b) suggesting that in the absence of p53 glycolysis was promoted. By normalization of various metabolites to tCr levels (notoriously increased during differentiation and then used as a reference control), we observed that a metabolic switch occurred when wild type myoblasts differentiated and it was mirrored by a decrease in the level of lactate in the last phases of differentiation (Fig. 24c). Differently, in p53 null cells the levels of lactate detected in myoblast and in myotubes were comparable (Fig. 24d). Moreover, in both wild type and p53 null cells, a decrease in phosphocolin (PCho) was observed during differentiation consistently with the decreased requirement of phospholipid synthesis in the last phase of chick embryo myogenesis (Granata D. R. et al., 2000). Based on these data, it is reasonable to suppose that during myogenic differentiation, which coincides with the increase

of mitochondrial biogenesis (Remels A. H. V. et al., 2010), the metabolic profile moves from the glycolytic pathway to an oxidative phenotype.

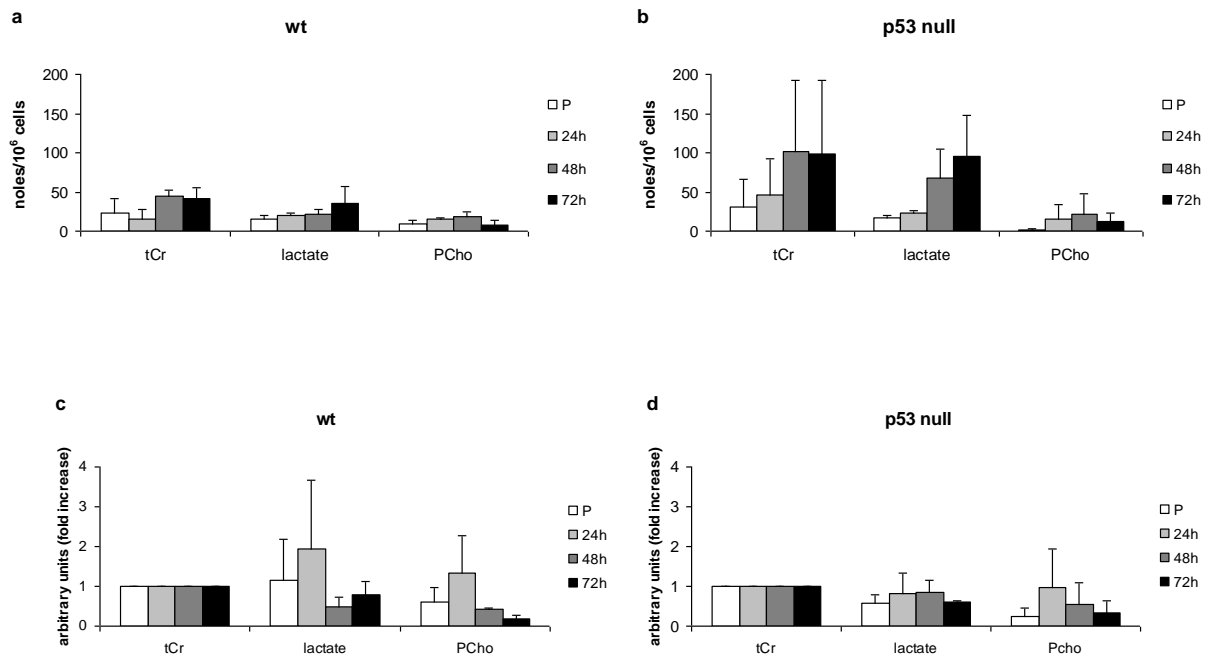


Figure 24. ¹H-NMR metabolic profile during differentiation. Quantification of aqueous metabolites (nmoles/10⁶ cells, n=3) by ¹H-NMR (nuclear magnetic resonance) in wild type (a) and p53 null (b) myoblasts (P) and myotubes (at 24, 48 and 72 h of differentiation). Metabolites: tCr (total creatine: creatine plus phosphocreatine); lactate; phosphocholine (PCho). Values of lactate and PCho normalized to tCr in wild type (c) and p53 null (d) myoblasts (P) and myotubes (at 24, 48 and 72 h of differentiation).

III. DISCUSSION

It is broadly accepted that the DNA repair capacity of post-mitotic cells differs from that of their proliferating counterpart. Terminally differentiated cells accumulate DNA damage due to the downregulation of the major DNA repair mechanisms but are resistant to various stressors (Fortini P. et al., 2012). Our study shed light on some of the mechanisms underlying this phenomenon in post-mitotic muscle cells. We show that ATM-mediated DDR is activated in myotubes by exposure to a variety of DNA damaging agents including SSB inducers but this is not sufficient to trigger cell death in post-mitotic cells. Specific types of damage such as DSB in transcriptionally active genes or induction of mitochondrial ROS (as induced by DOXO and MND, respectively) are required to activate p53 and the cell death pathway in these cells.

It was previously described that myotubes are defective in the repair processing of the abasic sites, which are very common lesions generated both spontaneously and as BER intermediates. At molecular level, BER impairment has been mainly ascribed to the strong downregulation of XRCC1 and the lack of DNA ligase I. The reduced BER capacity led to an accumulation of DNA lesions in myotubes versus myoblasts after exposure to hydrogen peroxide (Narciso L. et al., 2007). Further analysis revealed that other BER genes such as *Ung2* and *Myh* which code for uracil-DNA glycosylase2 (UNG2) and human mutY homolog (MYH) respectively, are strongly downregulated in post-mitotic muscle cells compared to the proliferating counterpart. This is in agreement with the role of these two enzymes in the removal of misincorporated bases during DNA replication. UNG2 deficiency has been confirmed at functional level testifying a clear impairment of this cell cycle-associated DNA glycosylase in terminally differentiated cells.

In order to better characterise the nature of the DNA lesions accumulated in myotubes after exposure to hydrogen peroxide, the analysis of DNA repair capacity was carried out after exposure to MMS, a pure inducer of alkylated bases which turn into SSB by BER processing. Similarly to what observed after oxidative DNA damage, a slower repair kinetics in myotubes compared to myoblasts after MMS exposure was observed testifying the accumulation of SSB in post-mitotic muscle cells.

In this study we provide the first evidence that in terminally differentiated cells SSB are able to activate ATM as the main kinase responsible for DDR triggering. Indeed it is largely accepted that DSB, either induced directly or as the result of unrepaired SSB at replication fork, initiate the DDR by activating the ATM kinase and DNA-PK which in turn activate the checkpoint kinase Chk2. On the other hand, ssDNA repair intermediates and ssDNA that arises during replication stress recruit the ATR complex (López-Contreras A. J. et al., 2010) that subsequently entails the activation of the checkpoint kinase Chk1. The ATR-Chk1 branch is absent in myotubes but we show that ATM activation and signalling by SSB occur also in this ATR-defective background. It has been

previously shown the activation of ATM-Chk2 in response to oxidative and alkylation damage in G₁ cells (Chou W. C. et al., 2008) and we observe that is also true in post-mitotic muscle cells. What are the lesions/events that activate ATM can be only a matter of speculation. The lack of involvement of ATM in the case of early appearing γ H2AX foci induced by MMS indicates that the canonical BER intermediates are unable to activate ATM. It has been proposed that these foci might represent regions of altered chromatin structure due to BER events (Stojic L. et al., 2004). In our cell system, ATM is involved in the late γ H2AX foci formation. Based on the alkylation pattern induced by MMS we may speculate that the lesions that are responsible for triggering the signalling cascade are produced by the unprocessed O⁶-alkylG/C mispairs. In proliferating cells the activation of ATM/ATR by O⁶-methyl-guanine requires a functional mismatch repair (Stojic L. et al., 2004) but what happens in post-mitotic cells that are supposed to be deprived of MMR, a postreplicative DNA repair mechanism, is an open question. Moreover, the lack of participation of DNA-PK to the H2AX dephosphorylation events upon H₂O₂ induced-damage indicates that NHEJ is not involved in their repair.

It has been previously shown that the radioresistance in myotubes is associated with a blockade of DNA damage signalling downstream of ATM (Latella L. et al., 2004). In this study we extend this investigation and we find that the resistance to SSB-induced cell death is also associated with defective signalling downstream of ATM. Upon SSB induction, p53 is not activated and its stabilization by nutlin-3 is not sufficient to trigger cell death. Similarly to myotubes, also neurons are resistant to a variety of genotoxic stresses. The so called restricted apoptosis of mature neurons has been ascribed to the reduction of the levels of Apaf-1 and to the associated increase in the protective activities of the inhibitors of the apoptosis proteins (IAPs) that sustain survival (Lindholm D. et al., 2004). DNA damage induced by topoisomerase2 inhibitor etoposide has been shown to overcome the inhibition by IAPs via a p53-mediated induction of Apaf-1 (Vaughn A. E. et al., 2007). A similar mechanism has been hypothesized for skeletal muscle cells (Smith M.I. et al., 2009). However, our data indicate that, after exposure to DNA break-inducing agents, both p53 stabilization and the associated stabilization of Apaf-1 (by nutlin-3) are not sufficient to trigger cell death. The type of damage and the involved cellular targets seem to play a key role in the activation of this mechanism. Indeed, post-mitotic muscle cells activate p53 upon exposure to the TOP2 inhibitor DOXO and to the mitochondria-specific inhibitor MND and die. After exposure to these compounds, p53 is stabilized and phosphorylated at serine 15 (activation of ATM leads to direct phosphorylation of p53 at serine 15) (Siliciano J. D. et al., 1997). This does not occur upon acute treatment of myotubes with H₂O₂, which induces nuclear ROS, suggesting that the functionality of mitochondria (and likely the activation of mitochondrial p53) play a key role in muscle integrity. The use of a ROS scavenger such as NAC fully protected myotubes from the toxic effects of MND indicating that oxidant stress at mitochondrial level is

involved in this response. What type of damage triggers cell death upon DOXO exposure is still unclear. Currently, the free radical hypothesis is most favoured due to the redox cycling ability of doxorubicin to generate highly reactive oxygen free radicals (Simůnek T., et al., 2009). However, we observe lack of modulation of DOXO toxicity by NAC supporting a model where the proteosomal processing of TOP2-DNA cleavage complexes induced by DOXO exposes DSB that, if not repaired, are the major contributors to cell death. Similar data have been obtained in cardiomyocytes (Lyu Y. L. et al., 2007) in which DOXO-induced DNA damage was shown to involve the TOP2 β isozyme that is the only TOP2 isoform expressed in adult heart .

Even if p53 is stabilized by DOXO and MND its function as transcription factor is not fully operational in myotubes as shown by the lack of p21 and Bax1 transactivation. Similarly, absent or modest activation of p53 target genes such as p21, MDM2 and Bax1 was reported in C2C12 myotubes exposed to DOXO (Latella L. et al., 2004). These findings suggest that the transcription-independent mitochondrial p53 program contributes to muscle cell death (Vaseva A. V. et al., 2009).

DNA-damage activated cell death may occur *via* different pathways including apoptosis, autophagy and necrosis. Autophagy may play a cell protective function as during differentiation but it can also cause cell death when it becomes over-activated or inhibited. We observed that autophagy is induced during *in vitro* muscle differentiation and is independent of the inactivation of mTOR, the negative regulator of autophagy. mTOR is a protein kinase and it is an essential component of two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Phosphorylation of mTORC1 mediated by Akt, leads to activation of mTORC1 which functions, in part, by phosphorylating and inhibiting Ulk1, thus preventing autophagy induction. Conversely, AMPK-mediated phosphorylation of raptor, a constituent of mTORC1, results in the inhibition of mTORC1 activity leading to autophagy. In contrast to the current view, it has been reported that autophagy can be induced independently of mTOR inhibition. Activation of insulin receptor substrate 2 (IRS-2), a scaffold protein that mediates the signalling cascades of growth factors such as insulin and insulin-like growth factor 1 (IGF-1), and threosine treatment are able to induce autophagy without inactivating mTOR in different cell lines (Yamamoto A. et al., 2006; Sarkar S. et al., 2006). In muscle, leucine starvation has been reported to induce mTOR-independent autophagy in cultured myotubes (Mordier S. et al., 2000) and it has been observed that during differentiation of C2C12 cells to myotubes the conjugation of GABARAP (one of the mammalian homolog of the yeast Atg8) is activated without inactivation of the mTOR kinase-signalling pathway (Tanida I. et al., 2006). The mechanism(s) underlying the induction of mTOR inactivation-independent autophagy is still unclear. Two recent studies demonstrate direct phosphorylation of Ulk1 by AMPK that leads to Ulk1 activation and autophagy induction (Egan D. F et al., 2011; Kim J. et al., 2011). So, we can speculate that

in our cell system AMPK may have a main role in the control of autophagy during differentiation without affecting mTOR activation. Consistently, when we trigger the autophagic pathway dependent on mTOR inactivation by rapamycin, we do not observe any effect on the efficiency of differentiation whereas an increase in the autophagic flux is detectable, suggesting that mTOR inactivation-dependent autophagy is functional in muscle cells although is not the pathway involved in basal autophagy and in differentiation.

Interestingly, although it has been described that p53 degradation is required for the induction of autophagy (Tasdemir E. et al., 2008), we observe that during muscle differentiation autophagy occurs also whether p53 is stabilized (by nutlin-3) thus preventing its physiological decrease during myotubes formation. In agreement with the hypothesis that p53 has not a role in the control of autophagy in our model, muscle cells derived from p53 null mice are able to complete the differentiation process and induce autophagy as well as p53-proficient cells. Similar data have been obtained in adipocytes which induce autophagy during differentiation in both a p53-proficient and -deficient background (Mikami K. et al., 2012).

It is possible that p53 exerts its inhibitory effect on autophagy only when the onset of this process is dependent on mTOR inactivation and this is the case of the autophagic pathway described in the study of Tasdemir and coworkers. Conversely, mTOR-inactivation-independent autophagy may have regulators other than p53.

In order to investigate the contribution of autophagy in the induction of muscle differentiation, we identify Beclin 1 as a good candidate for the interplay between autophagy and differentiation. Our data show that *Beclin 1* downregulation by siRNA delays the autophagic flux and affects the expression of the differentiation marker myosin, more precisely reducing the efficiency of myotubes fusion. So far, no data are available about the role of autophagy in the fusion process of myotubes, however our results indicate that basal autophagy observed during muscle differentiation is, at least partially, Beclin 1-dependent as well as the proper fusion of myotubes.

Finally, the cross-talk between p53 and metabolism is well characterized. p53 inhibits glycolysis at different steps inhibiting the expression of the glucose transporters GLUT1 and GLUT4 and decreasing the level of phosphoglycerate mutase (PGM) while increasing the expression of TIGAR which negatively regulates glycolysis (Schwartzberg-Bar-Yoseph F., et al., 2004; Bensaad K. et al., 2006). Consistently, we observe that the levels of lactate, a by-product of the anaerobic metabolism, are lower in wild type muscle cells when compared to p53-deficient cells. Moreover, a metabolic switch occurs when wild type muscle cells differentiate and parallels with reduced levels of lactate in the last phases of differentiation. This is in line with the increase in mitochondrial biogenesis and in the oxidative capacity observed during myogenesis (Remels A. H. V., et al., 2010; Leary S. C. et al., 1998) supporting the hypothesis that metabolism may shift from anaerobic glycolysis to oxidative phosphorylation.

It is intriguingly to address whether an impairment of the autophagic process during muscle differentiation may affect the metabolic profile typical of terminally differentiated cells.

IV. CONCLUSIONS

From a broader perspective our results indicate that the use of antioxidants is unlikely to prevent the cardiotoxicity of doxorubicin. Indeed, the effect of oxidative stress in clinical cardiotoxicity is increasingly questioned (Zhang Y.-W. et al., 2009). Our study identifies the activation of p53 by TOP2-linked DSB as the key event for the cytotoxic effect of doxorubicin in muscle cells.

The comprehension of the mechanisms underlying the cell death pathway and the characterization of basal autophagy in muscle cells may contribute to shed light on the process involved in several myopathies, such as Duchenne Muscular Dystrophy (DMD). Autophagy is emerging as an important process that limits muscle damage (Shintani T. and Klionsky D. J., 2004; Raben N. et al., 2008). Histo-pathological hallmarks of DMD, i.e. protein aggregation, presence of swollen and damaged mitochondria, as well as distension of sarcoplasmic reticulum, are often observed when autophagy is impaired (Millay D. P. et al., 2008; Culligan K. et al., 2002) and contribute to myofiber degeneration (Sandri M., 2010; Masiero E. et al., 2009). Very recent studies identify autophagy as a potential therapeutic target for ameliorating DMD (Pauly M. et al., 2012; De Palma C. et al., 2012) by removal of defective mitochondria and inhibition of muscle damage and inflammation, further highlighting the importance of this pathway in the maintenance of muscle integrity.

V. REFERENCES

Adams B. R., Hawkings A. J., Povirk L. F., Valerie K., ATM-independent, high-fidelity nonhomologous end joining predominates in human embryonic stem cells, *Aging*, 2010, 2, 582-596.

Alexander A., Cai S.L., Kim J., Nanez A., Sahin M., MacLean K.H., Inoki K., Guan K.L., Shen J., Person, D. Kusewitt, G.B. Mills, M.B. Kastan, C.L. Walker M.D., ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS, *Proc. Natl. Acad. Sci. U.S.A.*, 2010a, 107, 4153-4158.

Alexander A., Kim J., Walker C.L., ATM engages the TSC2/mTORC1 signaling node to regulate autophagy, *Autophagy*, 2010b, 6, 672-673.

Aymard E., Barruche V., Naves T., Bordes S., Closs B., Verdier M., Ratinaud M.H., Autophagy in human keratinocytes: an early step of the differentiation?, *Exp Dermatol.*, 2011, 20, 263-268.

Baerga R., Zhang Y., Chen P. H., Goldman S., Jin S., Targeted deletion of autophagy-related 5 (atg5) impairs adipogenesis in a cellular model and in mice, *Autophagy*, 2009, 5, 1118-1130.

Banin S., Moyal L., Shieh S., Taya Y., Anderson C. W., Chessa L., Smorodinsky N. I., Prives C., Reiss Y., Shiloh Y. and Ziv Y., Enhanced phosphorylation of p53 by ATM in response to DNA damage, *Science*, 1998, 281, 1674-1677.

Bensaad K., Tsuruta A., Selak M. A., Vidal M. N., Nakano K., Bartrons R., Gottlieb E., Vousden K. H., TIGAR, a p53-inducible regulator of glycolysis and apoptosis, *Cell*, 2006, 126, 107-120.

Belloni M., Uberti D., Rizzini C., Jiricny J., Memo M., Induction of two DNA mismatch repair proteins, MSH2 and MSH6, in differentiated human neuroblastoma SH-SY5Y cells exposed to doxorubicin, *J. Neurochem.*, 1999, 72, 974-979.

Bernstein K. A. and Rothstein R., At loose ends: resecting a double-strand break, *Cell*, 2009, 137, 807-810.

Biton S., DarI I., Mittelman L., Pereg Y., Barzilai A., Shiloh Y., Nuclear ataxia-telangiectasia mutated (ATM) mediates the cellular response to DNA double strand breaks in human neuron-like cells, *J. Biol. Chem.*, 2006, 281, 17482-17491.

Biton S., Gropp M., Itsykson P., Pereg Y., Mittelman L., Johe K., Reubinoff B., Shiloh Y., ATM-mediated response to DNA double strand breaks in human neurons derived from stem cells, *DNA Repair (Amst.)*, 2007, 6, 128-134.

Bjørkøy G., Lamark T., Brech A., Outzen H., Perander M., Øvervatn A., Stenmark H., and Johansen T., p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death, *J. Cell Biol.*, 2005, 171, 603–614.

Briegert M. and Kaina B., Human monocytes, but not dendritic cells derived from them, are defective in base excision repair and hypersensitive to methylating agents, *Cancer Res.*, 2007, 67, 26-31.

Canman C. E., Lim D. S., Cimprich K. A., Taya Y., Tamai K., Sakaguchi K., Appella E., Kastan M. B. and Siciliano J. D., Activation of the ATM kinase by ionizing radiation and phosphorylation of p53, *Science*, 1998, 281, 1677-1679.

Cecconi F. and Levine B., The role of autophagy in mammalian development: cell makeover rather than cell death, *Dev. Cell*, 2008, 15, 344-357

Chehab N. H., Malikzay A., Appel M. and Halazonetis D., Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53, *Genes Dev.*, 2000, 14, 278-288.

Chehab N. H., Malikzay A., Stavridi E. S. and Halazonetis T. D., Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, 96, 13777-13782.

Chinnaiyan A. M., The apoptosome: heart and soul of the cell death machine, *Neoplasia*, 1999, 1, 5-15.

Chou W. C., Wang H. C., Wong F. H., Ding S. L., Wu P. E., Shieh S. Y., Shen C. Y., Chk2-dependent phosphorylation of XRCC1 in the DNA damage response promotes base excision repair, *EMBO J.*, 2008, 27, 3140–3150.

Chowdhury D., Xu X., Zhong X., Ahmed F., Zhong J., Liao J., Dykxhoorn D. M., Weinstock D. M., Pfeifer G. P. and Lieberman J. A., PP4-phosphatase complex de phosphorylates gamma-H2AX generated during DNA replication, *Mol. Cell*, 2008, 31, 33-46.

Colussi C., Parlanti E., Degan P., Aquilina G., Barnes D., Macpherson P., Karran P., Crescenzi M., Dogliotti E., Bignami M., The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool, *Curr. Biol.*, 2002, 12, 912-918.

Cory S., and Adams J. M., The Bcl2 family: regulators of the cellular life-or-death switch, *Nat. Rev. Cancer*, 2002, 2, 647–656.

Culligan K., Banville N., Dowling P., Ohlendieck K., Drastic reduction of calsequestrin-like proteins and impaired calcium binding in dystrophic mdx muscle, *J. Appl. Physiol.*, 2002, 92, 435–445.

Dalal S. N., Schweitzer C. M., Gan J. and DeCaprio J. A., Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site, *Mol. Cell. Biol.*, 1999, 19, 4465-4479.

David P., Efrati E., Tocco G., Krauss S. W., Goodman M. F., DNA replication and postreplication mismatch repair in cell-free extracts from cultured human neuroblastoma and fibroblast cells, *J. Neurosci.*, 1997, 17, 8711–8720.

De Palma C., Morisi F., Cheli S., Pambianco S., Cappello V., Vezzoli M., Rovere-Querini P., Moggio M., Ripolone M., Francolini M., Sandri M., Clementi E., Autophagy as a new therapeutic target in Duchenne muscular dystrophy, *Cell Death Dis.*, 2012, 15, 3, e418

de Waard H., Sonneveld E., de Wit J., Esveldt-van Lange R., Hoeijmakers J. H., Vrieling H., van der Horst G. T. J., Cell-type-specific consequences of nucleotide excision repair deficiencies: embryonic stem cells versus fibroblasts, *DNA Repair (Amst.)*, 2008, 7, 1659-1669.

Deng C., Zhang P., Harper J. W., Elledge S. J., Leder P., Banin S., Moyal L., Shieh S., Taya Y., Anderson C. W., Chessa L., Smorodinsky N. I., Prives C., Reiss Y., Shiloh Y. and Ziv Y., Mice lacking p21/CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control, *Cell*, 1995, 82, 675-684.

Egan D. F., Shackelford D. B., Mihaylova M. M., Gelino S., Kohnz R. A., Mair W., Vasquez D. S., Joshi A., Gwinn D. M., Taylor R., Asara J. M., Fitzpatrick J., Dillin A., Viollet B., Kundu M., Hansen M., Shaw R. J., Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy, *Science*, 2011, 331,456–461.

Enari M., Sakahira H., Yokoyama H., Okawa K., Iwamatsu A., Nagata S., A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, *Nature*, 1998, 391, 43–50.

Fisher C. L. and Fisher A.G., Chromatin states in pluripotent, differentiated, and reprogrammed cells, *Curr. Opin. Genet. Dev.*, 2011, 21, 140–146.

Fortini P. and Dogliotti E., Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways, *DNA Repair (Amst.)*, 2007, 6, 398–409.

Fortini P. and Dogliotti E., Mechanisms of dealing with DNA damage in terminally differentiated cells, *Mutat. Res.*, 2010, 685, 38-44.

Fortini P., Ferretti C., Dogliotti E., The plasticity of DNA damage response during cell differentiation: pathways and consequences, *Issues in Toxicology No. 13, The Cellular Response to the Genotoxic Insult: The Question of Threshold for Genotoxic Carcinogens*, Edited by Helmut Greim and Richard J. Albertini © The Royal Society of Chemistry, 2012, 160-179.

Fousteri M. and Mullenders L. H., Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects, *Cell Research*, 2008, 18, 73–84.

Freeman A. K. and Monteiro A. N., Phosphatases in the cellular response to DNA damage, *Cell Commun. Signaling*, 2010, 8, 27-38.

Fulco M., Schiltz R. L., Iezzi S., King M. T., Zhao P., Kashiwaya Y., Hoffman E., Veech R. L., Sartorelli V., Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state, *Mol Cell.*, 2003, 12, 51-62.

Granata F., Iorio E., Carpinelli G., Giannini M., Podo F., Phosphocholine and phosphoethanolamine during chick embryo myogenesis: a ³¹P-NMR study, *Biochim. Biophys. Acta*, 2000, 1483, 334-342.

Green D. R. and Chipuk J. E., p53 and Metabolism: Inside the TIGAR, *Cell*, 2006, 126, 30-32.

Guo Z., Kumagai A., Wang S. X. and Dunphy W. G., Requirement for ATR in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts, *Genes Dev.*, 2000, 14, 2745-2756.

Ha H. C., Snyder S. H., Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, 96, 13978-13982.

Hakem R., DNA-damage repair; the good, the bad, and the ugly, *EMBO J.*, 2008, 27, 589-605.

Harfe B. D. and Robertson S. J., DNA Mismatch repair and Genetic Instability, *Ann. Rev. Gen.*, 2000, 34, 359-399.

Hill M. M., Adrain C., Duriez P. J., Creagh E. M., Martin S. J., Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes, *EMBO J.*, 2004, 23, 2134–2145.

Hirao A., Kong Y. Y., Matsuoka S., Wakeham A., Ruland J., Yoshida H., Liu D., Elledge S. J., Mak T. W., DNA damage-induced activation of p53 by the checkpoint kinase Chk2, *Science*, 2000, 287, 1824-1827.

Hoeijmakers J. H., DNA damage, aging, and cancer, *N. Engl. J. Med.*, 2009, 361, 1475-1485.

Hyka-Nouspikel N., Lemonidis K., Lu W. T., Nouspikel T., Circulating human B lymphocytes are deficient in nucleotide excision repair and accumulate mutations upon proliferation, *Blood*, 2011, 117, 6277-6286.

Ito H., Miller S. C., Billingham M. E., Akimoto H., Torti S. V., Wade R., Gahlmann R., Lyons G., Kedes L., Torti F. M., Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro, *Proc. Natl. Acad. Sci. U S A.*, 1990, 87, 4275-4279.

Jiricny J., The multifaceted mismatch-repair system, *Nat. Rev. Mol. Cell. Biol.*, 2006, 7, 335-346.

Joza N., Susin S. A., Daugas E., Stanford W. L., Cho S. K., Li C. Y., Sasaki T., Elia A. J., Cheng H. Y., Ravagnan L., Ferri K. F., Zamzami N., Wakeham A., Hakem R., Yoshida H., Kong Y. Y., Mak T. W., Zuniga-Pflucker J. C., Kroemer G., Penninger J. M., Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death, *Nature*, 2001, 410, 549–554.

Kenyon J. and Gerson S. L., The role of DNA damage repair in aging of adult stem cells, *Nucleic Acids Res.*, 2007, 35, 7557-7565.

Kerzendorfer C., O'Driscoll M., Human DNA damage response and repair deficiency syndromes: linking genomic instability and cell cycle checkpoint proficiency, *DNA Repair (Amst)*, 2009, 8, 1139-1152.

Kim J., Kundu M., Viollet B., Guan K.-L., AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, *Nat. Cell. Biol.*, 2011, 13, 132–141.

Kischkel F. C., Hellbardt S., Behrmann I., Germer M., Pawlita M., Krammer P. H., Peter M. E., Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor, *Embo J.*, 1995, 14, 5579–5588.

Kruman I. I., Wersto R. P., Cardozo-Pelaez F., Smilenov L., Chan S. L., Chrest F. J., Emokpae R. Jr, Gorospe M., Mattson M. P, Cell cycle activation linked to neuronal cell death initiated by DNA damage, *Neuron.*, 2004, 41, 549-561.

Kulkarni A., McNeill D. R., Gleichmann M., Mattson M. P., Wilson III D. M., XRCC1 protects against the lethality of induced oxidative DNA damage in nondividing neural cells, *Nucleic Acids Res.*, 2008, 36, 5111–5121.

Kundu M., Lindsten T., Yang C.-Y., Wu J., Zhao F., Zhang J., Selak M. A., Ney P. A., Thompson C. B., Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation, *Blood*, 2008, 112, 1493-1502.

Kuster G. M., Häuselmann S. P., Rosc- Schlüter B. I., Lorenz V., Pfister O., Reactive oxygen/nitrogen species and the myocardial cell homeostasis: an ambiguous relationship, *Antioxid. Redox Signal.*, 2010, 13, 1899–1910.

Latella L., Lukas J., Simone C., Puri P.L., Bartek J., Differentiation-induced radioresistance in muscle cells, *Mol. Cell. Biol.*, 2004, 24, 6350-6361.

Leary S. C., Battersby B. J., Hansford R. G., Moyes C. D., Interactions between bioenergetics and mitochondrial biogenesis, *Biochim. Biophys. Acta*, 1998, 1365, 22-30.

Lee Y., Katyal S., Li Y., El-Khamisy S. F., Russell H. R., Caldecott K. W., McKinnon P. J., Genesis of cerebellar interneurons and the prevention of neural DNA damage requires XRCC1, *Nat. Neurosci.*, 2009, 12, 973-980.

Li L. Y., Luo X., Wang X., Endonuclease G is an apoptotic DNase when released from mitochondria, *Nature*, 2001, 412, 95–99.

Lieber M. R., Lu H., Gu J., Schwarz K., Flexibility in the order of action and in the enzymology of the nuclease, polymerases, and ligase of vertebrate nonhomologous DNA end joining: relevance to cancer, aging, and the immune system, *Cell Res.*, 2008, 18, 125–133

Lin T., Chao C., Saito S., Mazur S.J., Murphy M.E., Appella E., Xu Y., p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression, *Nat. Cell Biol.*, 2005, 7, 165-171.

Lindholm D., Arumäe U., Cell differentiation: reciprocal regulation of Apaf-1 and the inhibitor of apoptosis proteins, *J. Cell. Biol.*, 2004, 167, 193–195.

Liu P., Qian L., Sung J. S., de Souza-Pinto N. C., Zheng L., Bogenhagen D. F., Bohr V. A., Wilson 3rd D. M., Shen B., Demple B., Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria, *Mol. Cell. Biol.*, 2008, 28, 4975–4987.

Liu Q., Guntuku S., Cui X. S., Matsuoka S., Cortez D., Tamai K., Luo G., Carattini-Rivera S., DeMayo F., Bradley A., Donehower L. A. and Elledge S. J., Chk1 is an essential kinase that is regulated by ATR and required for the G(2)/M DNA damage checkpoint, *Genes Dev.*, 2000, 14, 1448-1459.

Loor G., Kondapalli J., Schriever J. M., Chandel N. S., Vanden Hoek T. L., Schumacker P. T., Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis, *Free Radic. Biol. Med.*, 2010, 49, 1925–1936.

López-Contreras A. J., Fernandez-Capetillo O., The ATR barrier to replication-born DNA damage, *DNA Repair (Amst)*, 2010, 9, 1249–1255.

Löbrich M. and Jeggo P. A., The impact of a negligent G2/M checkpoint on genomic instability and cancer induction, *Nat. Rev. Cancer*, 2007, 7, 861-869.

Lukas C., Bartkova J., Latella L., Falck J., Mailand N., Schroeder T., Sehested M., Lukas J., Bartek J., DNA damage-activated kinase Chk2 is independent of proliferation or differentiation yet correlates with tissue biology, *Cancer Res*, 2001, 61, 4990–4993.

Lyu Y. L., Kerrigan J. E., Lin C. P., Azarova A. M., Tsai Y. C., Ban Y., Liu L. F., Topoisomerase IIbeta mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane, *Cancer Res.*, 2007, 67, 8839–8846.

Ma X. M. and Blenis J., Molecular mechanisms of mTOR-mediated translational control, *Nat. Rev. Mol. Cell. Biol.*, 2009, 10, 307-318.

Macpherson P., Barone F., Maga G., Mazzei F., Karran P., Bignami M., 8-oxoguanine incorporation into DNA repeats in vitro and mismatch recognition by MutSalpha. *Nucleic Acids Res.*, 2005, 33, 5094-5105.

Mariño G., Madeo F., Kroemer G., Autophagy for tissue homeostasis and neuroprotection, *Curr. Opin. Cell. Biol.*, 2011, 23, 198-206.

Masiero E., Agatea L., Mammucari C., Blaauw B., Loro E., Komatsu M., Metzger D., Reggiani C., Schiaffino S., Sandri M., Autophagy is required to maintain muscle mass, *Cell Metab.*, 2009, 10, 507–515.

Matsuoka S., Rotman G., Ogawa A., Shiloh Y., Tamai K. and Elledge S. J., Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 10389-10394.

Maynard S., Swistikowa A. M., Lee J. W., Liu J., Liu S.-T., Da Cruz A., Rao M., de Souza-Pinto N., Zeng X., Bohr V. A., Human embryonic stem cells have enhanced repair of multiple forms of DNA damage, *Stem Cells*, 2008, 26, 2266-2274.

McVey M., Lee S. E., MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings, *Trends Genet.*, 2008, 24, 529–538.

Meulle A., Salles B., Daviaud D., Valet P., Muller C., Positive regulation of DNA double strand break repair activity during differentiation of long life span cells: the example of adipogenesis, *PLoS One*, 2008, 3, e3345.

Mikami K., Okita N., Tokunaga Y., Ichikawa T., Okazaki T., Takemoto K., Nagai W., Matsushima S., Higami Y., Autophagosomes accumulate in differentiated and hypertrophic adipocytes in a p53-independent manner, *Biochem. Biophys. Res. Commun.*, 2012, 427, 758-763.

Millay D. P., Sargent M. A., Osinska H., Baines C. P., Barton E. R., Vuagniaux G., Sweeney H. L., Robbins J., Molkentin J. D., Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy, *Nat. Med.*, 2008, 14, 442–447.

Misteli T. and Soutoglou E., The emerging role of nuclear architecture in DNA repair and genome maintenance, *Nat. Rev. Mol. Cell. Biol.*, 2009, 10, 243-254.

Mizushima N. and Levine B., Autophagy in mammalian development and differentiation, *Nat. Cell. Biol.*, 2010, 12, 823-830.

Mizushima N., Levine B., Cuervo A. M., Klionsky D. J., Autophagy fights disease through cellular self-digestion, *Nature*, 2008, 451, 1069-1075.

Mohrin M., Bourke E., Alexander D., Warr M. R., Barry-Holson K., Le Beau M. M., Morrison C. G., Passegué E., Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis, *Cell Stem Cell*, 2010, 7, 174-185.

Mordier S., Deval C., Bechet D., Tassa A., Ferrara M., Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway, *J. Biol. Chem.*, 2000, 275, 29900–29906.

Muñoz-Gómez J. A., Rodríguez-Vargas J. M., Quiles-Pérez R., Aguilar-Quesada R., Martín-Oliva D., de Murcia G., Menissier de Murcia J., Almodros A., Ruiz de Almodóvar M., Oliver F. J., PARP-1 is involved in autophagy induced by DNA damage, *Autophagy*, 2009, 5, 61-74.

Naka K. and Hirao A., Maintenance of genomic integrity in hematopoietic stem cells, *Int. J. Hematol.*, 2011, 93, 434-439.

Nakada S., Chen G. I., Gingras A. C., Durocher D., PP4 is a gamma H2AX phosphatase required for recovery from the DNA damage checkpoint, *EMBO Rep.*, 2008, 9, 1019-1026.

Narciso L., Fortini P., Pajalunga D., Franchitto A., Liu P., Degan P., Frechet M., Demple B., Crescenzi M., Dogliotti E., Terminally differentiated muscle cells are defective in base excision DNA repair and hypersensitive to oxygen injury, *Proc. Natl. Acad. Sci. U.S.A.*, 2007, 104, 17010-17015.

Nithipongvanitch R., Ittarat W., Cole M. P., Tangpong J., Clair D. K., Oberley T. D., Mitochondrial and nuclear p53 localization in cardiomyocytes: redox modulation by doxorubicin (Adriamycin)?, *Antioxid. Redox Signal.*, 2007, 9, 1001–1008.

Nospikel T. and Hanawalt P. C., Impaired nucleotide excision repair upon macrophage differentiation is corrected by E1 ubiquitin-activating enzyme, *Proc. Natl. Acad. Sci. U.S.A.*, 2006, 103, 16188–16193.

Nospikel T., DNA repair in differentiated cells: some new answers to old questions, *Neuroscience*, 2007, 145, 1213–1221.

Nospikel T., DNA repair in mammalian cells: so DNA repair really is that important?, *Cell. Mol. Life Sci.*, 2009a, 66, 965–967.

Nospikel T., Nucleotide excision repair: variations on versatility, *Cell. Mol. Life Sci.*, 2009b, 66, 994-1009.

Nussenzweig A., Nussenzweig M. C., A backup DNA repair pathway moves to the forefront, *Cell*, 2007, 131, 223–225.

Orii K. E., Lee Y., Kondo N., McKinnon P. J., Selective utilization of non homologous end-joining and homologous recombination DNA repair pathways during nervous system development, *Proc. Natl. Acad. Sci. U.S.A.*, 2006, 103, 10017–10022.

Pajalunga D., Mazzola A., Salzano A. M., Biferi M. G., De Luca G., Crescenzi M., Critical requirement for cell cycle inhibitors in sustaining nonproliferative states, *J. Cell Biol.*, 2007, 176, 807–818.

Pallis A. G. and Karamouzis M. V., DNA repair pathways and their implications in cancer treatment, *Cancer Metastasis Rev.*, 2010, 29, 677-685.

Panigrahi G. B., Lau R., Montgomery S. E., Leonard M. R., Pearson C. E., Slipped (CTG)ⁿ(CAG)^m repeats can be correctly repaired, escape repair or undergo errorprone repair, *Nat. Struct. Mol. Biol.*, 2005, 12, 654–662.

Paull T.T. and Lee J. H., The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM, *Cell Cycle*, 2005, 4, 737-740.

Pauly M., Daussin F., Burelle Y., Li T., Godin R., Fauconnier J., Koechlin-Ramonatxo C., Hugon G., Lacampagne A., Coisy-Quivy M., Liang F., Hussain S., Matecki S., Petrof B. J., AMPK activation stimulates autophagy and ameliorates muscular dystrophy in the mdx mouse diaphragm, *Am. J. Pathol.*, 2012, 181, 583-592.

Peng C. Y., Graves P. R., Thoma R. S., Wu Z., Shaw A. S., Piwnicka-Worms H., Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216, *Science*, 1997, 277, 1501-1505.

Petak I., Tillman D. M., Houghton J. A., p53 dependence of Fas induction and acute apoptosis in response to 5-fluorouracil-leucovorin in human colon carcinoma cell lines, *Clin. Cancer Res.*, 2000, 6, 4432-4441.

Picard F., Kurtev M., Chung N., Topark-Ngarm A., Senawong T., Machado De Oliveira R., Leid M., McBurney M. W., Guarente L., Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma, *Nature*, 2004, 429, 771-776.

Pitchard U., Watson A. J. M., Apoptosis and Gastrointestinal pharmacology, *Pharmacol. Ther.*, 1996, 72, 149-169.

Pommier Y., Leo E., Zhang H., Marchand C., DNA topoisomerases and their poisoning by anticancer and antibacterial drugs, *Chem. Biol.*, 2010, 17, 421–433.

Puri P.L., Bhakta K., Wood L.D., Costanzo A., Zhu J., Wang J.Y., A myogenic differentiation checkpoint activated by genotoxic stress, *Nat Genet.*, 2002, 32, 585-593.

Puri P. L. and Sartorelli V., Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications, *J. Cell Physiol.*, 2000, 185, 155–173.

Remels A. H. V., Langen R. C. J., Schrauwen P., Schaart G., Schols A. M. W. J., Gosker H. R., Regulation of mitochondrial biogenesis during myogenesis, *Mol. Cell. Endocrinol.*, 2010, 315, 113-120.

Raben N., Hill V., Shea L., Takikita S., Baum R., Mizushima N., Ralston E., Plotz P., Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated proteins and their potential role in muscle damage in Pompe disease, *Hum. Mol. Genet.*, 2008, 17, 3897–3908.

Robert T., Vanoli F., Chiolo I., Shubassi G., Bernstein K. A., Rothstein R., Botrugno O. A., Parazzoli D., Oldani A., Minucci S., Foiani M., HDACs link the DNA damage response, processing of double-strand breaks and autophagy, *Nature*, 2011, 471, 74-79.

Rossi D. J., Bryder D., Seita J., Nussenzweig A., Hoeijmakers J., Weissman I. L., Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age, *Nature*, 2007, 447, 725-729.

Saelens X., Festjens N., Vande Walle L., van Gorp M., van Loo G., Vandenabeele P., Toxic proteins released from mitochondria in cell death, *Oncogene*, 2004, 23, 2861–2874.

Sanchez Y., Wong C., Thoma R. S., Richman R., Wu Z., Piwnicka-Worms H., Elledge S. J., Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25, *Science*, 1997, 277, 1497-1501.

Sandri M., Autophagy in skeletal muscle, *FEBS Lett.*, 2010, 584, 1411–1416.

Sarkar S., Davies J.E., Huang Z., Tunnacliffe A., Rubinsztein D.C., Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein, *J. Biol. Chem.*, 2007, 282, 5641–5652.

Savitz S. I., Daniel B. A., Rosenbaum M. D., Apoptosis in neurological disease, *Neurosurgery*, 1998, 42, 555-572.

Schmetsdorf S., Arnold E., Holzerm M., Arendt T., Gärtner U., A putative role for cell cycle-related proteins in microtubule-based neuroplasticity, *Eur. J. Neurosci.*, 2009, 29, 1096- 1107.

Schneider L., Fumagalli M., d'Adda di Fagagna F., Terminally differentiated astrocytes lack DNA damage response signaling and are radioresistant but retain DNA repair proficiency, *Cell Death Differ.*, 2012, 19, 582-591.

Schwartzenberg-Bar-Yoseph F., Armoni M., Karnieli E., The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression, *Cancer Res.*, 2004, 64, 2627-2633.

Seita J., Rossi D. J., Weissman I. L., Differential DNA damage response in stem and progenitor cells, *Cell Stem Cell*, 2010, 7, 145-147.

Shangary S. and Wang S., Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy, *Annu. Rev. Pharmacol. Toxicol.*, 2009, 49, 223–241.

Shi X., Garry D. J., Muscle stem cells in development, regeneration, and disease, *Genes Dev.*, 2006, 20, 1692-1708.

Shieh S. Y., Ahn J., Tamai K., Taya Y. and Prives C., The human homologs of checkpoint kinase Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites, *Genes Dev.*, 2000, 14, 289-300.

Shiloh Y., ATM: expanding roles in the DNA damage response and cellular homeostasis, *Biochem. Soc. Trans.*, 2001, 29, 661-666.

Shintani T. and Klionsky D. J., Autophagy in health and disease: a double-edged sword, *Science*, 2004, 306, 990–995.

Siliciano J. D., Canman C. E., Taya Y., Sakaguchi K., Appella E., Kastan M. B., DNA damage induces phosphorylation of the amino terminus of p53, *Genes Dev.*, 1997, 11, 3471–3481.

Simonatto M., Giordani L., Marullo F., Minetti G. C., Puri P. L., Latella L., Coordination of cell cycle, DNA repair and muscle gene expression in myoblasts exposed to genotoxic stress, *Cell Cycle*, 2011, 10, 2355-2363.

Simůnek T., Stérba M., Popelová O., Adamcová M., Hrdina R., Gersl V., Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron, *Pharmacol. Rep.*, 2009, 61, 154–171.

Smith M. I., Huang Y. Y., Deshmukh M., Skeletal muscle differentiation evokes endogenous XIAP to restrict the apoptotic pathway, *PLoS One*, 2009, 4, e5097.

Stojic L., Mojas N., Cejka P., di Pietro M., Ferrari S., Marra G., Jiricny J., Mismatch-repair-dependent G2 checkpoint induced by low doses of SN1 type methylating agents requires the ATR kinase, *Genes Dev.*, 2004, 18, 1331–1344.

Stucki M. and Jackson S. P., MDC1/NFBD1: a key regulator of the DNA damage response in higher eukaryotes, *DNA repair*, 2004, 3, 953-957.

Sung J. S., DeMott M. S., Demple B., Long-patch base excision DNA repair of 2-deoxyribonolactone prevents the formation of DNA-protein cross-links with DNA polymerase beta, *J. Biol. Chem.*, 2005, 280, 39095–39103.

Szczesny B., Tann A. W, Longley M. J., Copeland W. C., Mitra S., Long patch base excision repair in mammalian mitochondrial genomes, *J. Biol. Chem.*, 2008, 283, 26349–26356.

Tanida I., Wakabayashi M., Kanematsu T., Minematsu-Ikeguchi N., Sou Y. S., Hirata M., Ueno T., Kominami E., Lysosomal turnover of GABARAP-phospholipid conjugate is activated during differentiation of C2C12 cells to myotubes without inactivation of the mTor kinase-signaling pathway, *Autophagy*, 2006, 264-271.

Tasdemir E., Maiuri M. C., Galluzzi L., Vitale I., Djavaheri-Mergny M., D'Amelio M., Criollo A., Morselli E., Zhu C., Harper F., Nannmark U., Samara C., Pinton P., Vicencio J. M., Carnuccio R., Moll U. M., Madeo F., Paterlini-Brechot P., Rizzuto R., Szabadkai G., Pierron G., Blomgren K., Tavernarakis N., Codogno P., Cecconi F., Kroemer G., Regulation of autophagy by cytoplasmic p53, *Nat. Cell. Biol.*, 2008, 10, 676-687.

Tichy E. D., Liang L., Deng L., Tischfield J., Schwemberger S. J., Babcock G. F., Stambrook P. J., Mismatch and base excision repair proficiency in murine embryonic stem cells, *DNA repair (Amst)*, 2011, 10, 445-51.

Tichy E. D., Pillai R., Deng L., Liang L., Tischfield J., Schwemberger S. J., Babcock G. F., Stambrook P. J., Mouse embryonic stem cells, but not somatic cells, predominantly use homologous recombination to repair double-strand DNA breaks, *Stem cells Dev.*, 2010, 19, 1699-16711.

Tichy E.D. and Stambrook P. J., DNA repair in murine embryonic stem cells and differentiated cells, *Exp. Cell. Res.*, 2008, 314, 1929–1936.

Tomashevski A., Webster D. R., Grammas P., Gorospe M., Kruman I.I., Cyclin C-dependent cell cycle entry is required for activation of nonhomologous end joining DNA repair in postmitotic neurons, *Cell Death Differ.*, 2010, 17, 1189-1198.

Unger T., Juven-Gershon T., Moallem E., Berger M., Vogt Sionov R., Lozano G., Oren M. and Haupt Y., Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2, *EMBO J.*, 1999, 18, 1805-1814.

Vaseva A. V., Moll U. M., The mitochondrial p53 pathway, *Biochim. Biophys. Acta*, 2009, 1787, 414-420.

Vaughn A. E., Deshmukh M., Essential postmitochondrial function of p53 uncovered in DNA damage-induced apoptosis in neurons, *Cell Death Differ.*, 2007, 14, 973-981.

Vousden K. H. and Ryan K. M., p53 and metabolism, *Nat. Rev. Cancer*, 2009, 9, 691-700.

Wallingford J. B., Seufert D. W., Virta V. C., Vize P. D., p53 activity is essential for normal development in *Xenopus*, *Curr. Biol.*, 1997, 7, 747-757.

Wang B., Matsuoka S., Carpenter P. B., Elledge S. J., 53BP1, a mediator of the DNA damage checkpoint, *Science*, 2002, 298, 1435-1438.

Wang H., Perrault A. R., Takeda Y., Qin W., Iliakis G., Biochemical evidence for Ku-independent backup pathways of NHEJ, *Nucleic Acids Res.*, 2003, 31, 5377-5388.

Ward I. and Chen J., Early events in the DNA damage response, *Curr. Topics Dev. Biol.*, 2004, 63, 1-35.

Weil M., Coles J. H. R. S., Davies T. J., Gardner R. L., Raff K. D., Raff M. C., Constitutive expression of the machinery for programmed cell death, *J. Cell Biol.*, 1996, 133, 1053-1059.

White E., Life, death, and the pursuit of apoptosis, *Genes Dev.*, 1996, 10, 1-15.

Yamamoto A., Cremona M. L., Rothman J.E., Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway, *J. Cell Biol.*, 2006, 172, 719-731.

Zhang A., Lyu Y. L., Lin C. P., Zhou N., Azarova A. M., Wood L. M., Liu L. F., A protease pathway for the repair of topoisomerase II-DNA covalent complexes, *J. Biol. Chem.*, 2006, 281, 35997–36003.

Zhang Y., Qu D., Morris E. J., O'Hare M. J., Callaghan S. M., Slack R. S., Geller H. M., Park D. S., The Chk1/Cdc25A pathway as activators of the cell cycle in neuronal death induced by camptothecin, *J. Neurosci.*, 2006, 26, 8819-8828.

Zhang Y.-W., Shi J., Li Y.-J., Wei L., Cardiomyocyte death in doxorubicin-induced cardiotoxicity, *Arch. Immunol. Ther. Exp.*, 2009, 57, 435–445.

Zhou B. B., Chaturvedi P., Spring K., Scott S. P., Johanson R. A., Mishra R., Mattern M. R., Winkler J. D., Khanna K. K., Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity, *J. Biol. Chem.*, 2000, 275, 10342-10348.

ACKNOWLEDGEMENTS

This study was carried out in the laboratory of Molecular Epidemiology, Department of Environmental and Primary Prevention, Istituto Superiore di Sanità.

I want to thank my supervisor, Dott.ssa Paola Fortini and the head of the laboratory, Dott.ssa Eugenia Dogliotti for their scientific support.

I would like to thank Prof. Antonio Antocchia for his useful suggestions.

I am grateful to Barbara and Lori who have always been when I needed them.

I would also like to thank Francesca and Piera who share with me the last year of this experience. It was a pleasure for me to meet two beautiful people like them.

A very special thanks is for my colleagues who became my friends, Sara, Ilenia and Vitalba. Without their presence my PhD would not have been the same and they know it.

Despite the thousands of miles that separate us, I cannot forget the strong support of my dearest friend Francesca. In moments like this one I regret that she does not live where I live.

A grateful thinking to the people who have always believed in me and they always will, my family. It is also thanks to them that I have achieved this.

The biggest thanks goes to the person who is closest to me, everyday. He has encouraged me, supported in difficult times and has always managed to make me smile back and drive.