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Role of MAGEA and MAGED2 proteins in genotoxic stress resistance and in DNA damage response

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ROLE OF MAGEA AND MAGED2 PROTEINS IN GENOTOXIC STRESS RESISTANCE AND IN DNA DAMAGE RESPONSE

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Role of MAGEA and MAGED2 proteins in genotoxic stress resistance and in DNA damage response

CARTE Pauline

The genetic material can suffer a variety of damages that can be caused by endogenous and exogenous damaging agents. Lesions on one of the DNA strand are repaired by mismatch repair, nucleotide or base excision repair or bypassed by translesion synthesis. DNA double strand break are mainly repaired by Non Homologous End Joining and homologous recombination. Recent studies have shown that some Melanoma Antigen-encoding (MAGE) proteins are involved in DNA damage repair. Those proteins are part of a family of more than 50 genes in Human and is divided into two classes, type I MAGEs (MAGEA, B and C) and type II MAGEs (MAGED, E, F, G, H, L and Necdin). MAGEA4 promotes translesion synthesis, a pathway that allows DNA replication across DNA lesion. MAGEC2 facilitates DNA double strand break repair through the phosphorylation of TRIM28, an E3 ubiquitin ligase, on 824 serine by the ataxia telangiectasia-mutated protein *kinase (ATM)*. MAGEG1 is part of the Structural maintenance of chromosomes 5-6 complex and is essential for homologous recombination. MAGED2 has been shown to delocalize after genotoxic stress and it modulates DNA damage response. Our objective was to further investigate the role of MAGEA and MAGED2 proteins in genotoxic stress resistance and DNA damage response in MelJuso cells.

We observed that MAGEA-depleted cells were more sensitive to camptothecin, a topoisomerase I inhibitor, compared to control cells. We also looked at the phosphorylation of histone H2AX, a early marker of DNA double-strand break, during camptothecin treatment and after release from drug. We observed a much stronger phosphorylation of H2AX during camptothecin treatment and, after removing the drug, seemed to disappeare more slowly in cells depleted for MAGEA. All these researches were also carried out in p53-KO MelJuso cells that we had obtained in our laboratory by CRISPR/Cas9 technology to determine whether or not MAGEA's role depends on p53. We also assessed the role of MAGED2 in genotoxic stress resistance and DNA damage response in context of camptothecin treatment. We observed that MAGED2-depleted cells were more sensitive to camptothecin. Taken together our results show that MAGEA and MAGED2 depletion impact the cell resistance to camptothecin and that these proteins could play a role in the early events of DNA damage response or in DNA double strand break repair, which still remains to be confirmed.

Mémoire de master en sciences biologiques

Décembre 2018

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Rôle des protéines MAGEA et MAGED2 dans la résistance au stress génotoxique et dans la réponse aux dommages de l'ADN

CARTE Pauline

Le génome peut subir une variété de dommages qui peuvent être causés par des agents endogènes et exogènes. Les lésions sur l'un des brins d'ADN sont réparées par la voie de réparation des mésappariement, par excision de base ou de nucléotide ou sont contournées par la synthèse translésionnelle. Les cassures double-brin de l'ADN sont principalement réparées par recombinaison. Des études récentes ont montré que certaines protéines MAGE sont impliquées dans la réparation des dommages de l'ADN. Ces protéines font partie d'une famille de plus de 50 gènes chez l'homme et sont divisées en deux classes, les MAGE de type I (MAGEA, B et C) et de type II (MAGED, E, F, G, H, L et Necdin). MAGEA4 favorise la synthèse translésionnelle, une voie qui permet la réplication de l'ADN à travers certaines lésions de l'ADN. MAGEC2 facilite la réparation des cassures double-brin d'ADN par la phosphorylation de TRIM28, une ubiquitine ligase E3, sur sa serine 824 par la protéine kinase ATM. MAGEG1 fait partie du complexe d'entretien structural des chromosomes (SMC 5-6) et est essentiel pour la recombinaison homologue. MAGED2, quant à lui, délocalise après un stress génotoxique et module la réponse aux dommages de l'ADN. Notre objectif était d'étudier le rôle de MAGEA et de MAGED2 dans la résistance au stress génotoxique et la réponse aux dommages à l'ADN dans les cellules MelJuso.
Nous avons observé que les cellules appauvries en MAGEA étaient plus sensibles à la camptothécine, un inhibiteur de la topoisomérase I, que les cellules control. Nous avons également étudié la phosphorylation de l'histone H2AX, un marqueur précoce des cassures double-brin de l'ADN, pendant le traitement à la camptothécine et après retrait de celle-ci. Nous avons observé une forte phosphorylation de H2AX lors du traitement et, après avoir enlevé la camptothécine, ce marqueur de cassure semblait disparaître plus lentement dans les cellules appauvries en MAGEA. Toutes ces recherches ont également été réalisées sur des cellules MelJuso p53-KO afin de déterminer si le rôle de MAGEA dépend ou non de p53. Nous avons également étudié le rôle de MAGED2 dans la résistance au stress génotoxique et la réponse aux dommages à l'ADN. Nous avons observé que les cellules appauvries en MAGED2 étaient plus sensibles à la camptothécine. L'ensemble de nos résultats montre que l'appauvrissement en MAGEA et MAGED2 a un impact sur la résistance cellulaire à la camptothécine et que ces protéines pourraient jouer un rôle dans la réponse ou dans la réparation des dommages à l'ADN, mais cela doit rester encore à être confirmer.

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LIST OF ABBREVIATION

Alt-EJ	Alternative End Joing
AMPK	AMP activated protein kinase
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3 related
BER	Base Excision Repair
BRC1	Breast Cancer type 1
BRC2	Breast Cancer type 2
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
CPT	Camptothecin
DDR	DNA Damage Response
DNA	Deoxyribo Nucleic Acid
DDSB	DNA Double Strand Break
FBP1	Fructose-1,6-biphosphate
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H2AX	Histone 2A member X
HR	Homologous Recombination
MAGE	Melanoma Antigen Gene
MHD	MAGE Homology Domain
MMR	Mismatch Repair
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NSE	Non-SMC Element
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffer Saline
PI3K	Phosphatidylinositol 3-kinase
Pol	Polymerase
RING	Really Interesting New Gene
RNA	Ribo Nucleic Acid

RPA	Replication Protein A
siRNA	Short interfering Ribo Nucleic Acid
SMC	Structural Maintenance of Chromosome
SSA	Single Strand Annealing
SSB	Single strand break
ssDNA	Single-strand DNA
TDP1	Tyrosyl-DNA phosphodiesterase 1
Topo-I	Topoisomerase-I DNA
TLS	Translesion synthesis
TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultra-Violet

I. INTRODUCTION

1. DNA damages

The genetic material can suffer a variety of damages that can be caused by endogenous and exogenous damaging agents. Endogenous sources of DNA damages include spontaneous chemical reactions, such as oxidation, hydrolysis and alkylation and endogenous agents, such as reactive oxygen and nitrogen species, lipid peroxidation products, alkylating agents, estrogen and cholesterol metabolites and reactive carbonyl species. Errors can also occur during replication such as when one or more bases are mis-incorporated. Exogenous physical and chemical agents include ionizing radiation, ultraviolet (UV) radiation and a wide variety of other chemicals agents. The types of damage are also multiple: insertion or deletion of a nucleotide, single strand break, double-strand break, mismatch of DNA bases, creation of photoproducts, inter and intra-strand crosslinking, chemical modifications, oxidative damage, etc. These damages can lead to a loss of DNA integrity that can result in severe diseases such as neurological disorders and cancer (Hakem, R. 2008).

1.1. DNA damage induced by camptothecin

Camptothecin (CPT) is a plant alkaloid identified as an anticancer agent which induces DNA-damages. It inhibits the topoisomerase-I DNA (topo-I), a nuclear enzyme that regulate the topological structure of DNA during DNA replication and transcription. This enzyme binds to DNA through a phosphotyrosine bound. Then it induces a reversible single-strand break that allows relaxation of supercoiled DNA and, eventually, catalyzes the DNA religation (Champoux, J.J. 2001). Camptothecin interposes between topo-I and DNA and inhibits the rejoining step, resulting in accumulation of covalent intermediate complex, the topo-I cleavable complex (Figure 1) (Hsiang, Y.-H. *et al.* 1988, Hsiang, Y.-H. *et al.* 1989, Hsiang, Y.H. *et al.* 1985, Legarza, K. *et al.* 2006, Legarza, K. *et al.* 2006).

Camptothecin is mostly toxic during S-phase, when the topo-I cleavable complex collides with the replication forks. Three events involved in cell death appear following this collision: arrest of the replication fork, formation of topo-I-linked DNA break at the site of collision and formation of a DNA double-strand break (DDSB). The DDSB is the main cause of the cytotoxic effect of CPT. Left unrepaired, this type of lesion can be responsible for G2-phase arrest (F. Liu, L. *et al.* 2000). Another effect of camptothecin is the arrest of RNA synthesis at the level of the elongation. The topo-I cleavable complex, located on the template strand, can collide with the RNA polymerase leading to conversion of this cleavable complex into single-strand breaks (Wu, J. *et al.* 1997).

A cell response to CPT-induced damage involved the dissociation of the topo-I cleavable complex. Indeed, after the collision, the ubiquitin/26S proteasome pathway is activated. Topo-I is polyubiquitinated and partially degraded by the 26S proteasome (Lin, C.P. *et al.* 2008). Then, tyrosyl-DNA phosphodiesterase 1 (TDP1) can access topo-I and hydrolyze the phosphotyrosyl bond between the DNA and the enzyme fragment. This hydrolysis results in the formation of DNA single-strand break (SSB) with a 3′ phosphate end and a 5′ hydroxyl end. These ends are then be processed by the bifunctional polynucleotide kinase 3′-phosphatase (PNKP) leading to dephosphorylation of the 3′ end and phosphorylation of the 5′ DNA end. Then the canonical SSB repair factors such as poly(ADP-ribose) polymerase 1 (PARP1), X-ray repair cross-complementing protein 1 (XRCC1) and DNA ligase 3 (LIG3) religate the break and complete the repair (Stingele, J. *et al.* 2017).

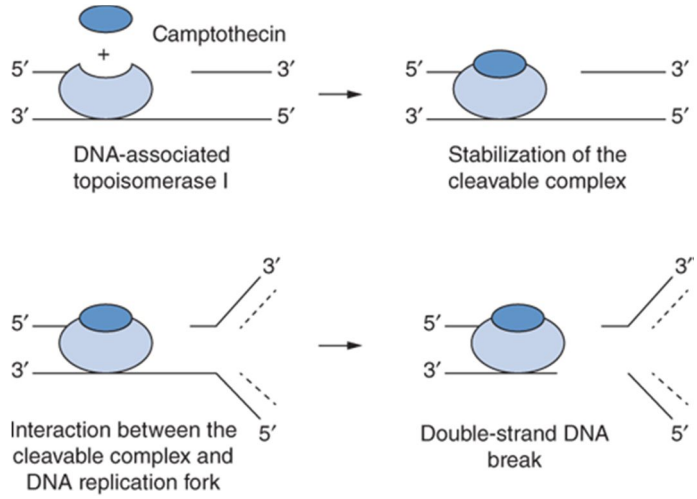

Figure 1. Mechanism of action of Camptothecin. From Harrison's Manual of Oncology, Bruce, A. et al.

2. Repair Pathways

Regardless of the damage agent and the kind of damage induced, DNA integrity and stability must be preserved to avoid diseases. That is why numerous systems of DNA repair have been developed. There are simple repair systems, which means that damage will be repaired by a specific enzyme, and complex/multi-step systems grouping Mismatch repair (MMR), Nucleotide Excision Repair (NER), Base Excision Repair (BER), Non Homologous End-Joining (NHEJ), Homologous recombination (HR), Single Strand Annealing (SSA), Alternative End Joining (alt-EJ) and Translesion synthesis (TLS) (Figure 2) (Hakem, R. 2008, Hoeijmakers, J.H. 2009).

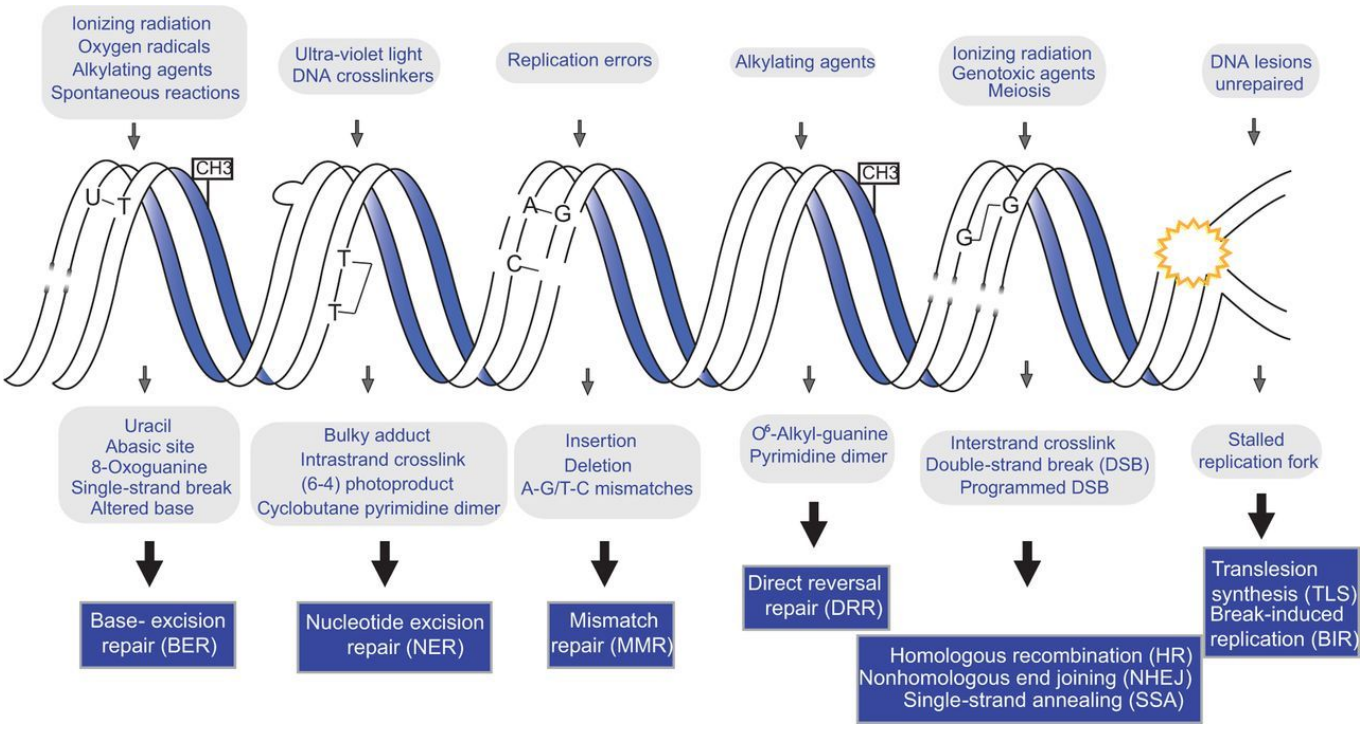

Figure 2. DNA damages and the corresponding DNA damages repair pathways. DNA can be targeted by different damaging agents leading to different types of damage. There are different DNA repair pathways specific to the type of damage including Base Excision Repair, Nucleotide Excision Repair, Mismatch Repair, Direct Reversal Repair, Homologous Recombination, Non Homologous End Joining, Single Strand Annealing, Alternative End Joining and Trans-lesion synthesis (TLS). From the Microbiology and Molecular Biology Reviews, Genois, M-M. et al, 2014.

The MMR repairs small insertions, deletions and mis-incorporation of bases which appear during replication. The NER allows to remove a damaged nucleotide from the DNA while the BER removes a damaged base. The NHEJ, HR, SSA and the alt-EJ are pathway allowing the repair of DDSB. TLS is a damage tolerance pathway that allows DNA replication across DNA lesion such as pyrimidine dimmers or apurinic site which stall the movement of replication fork (Hakem, R. 2008).

2.1. Repair of DNA double-strand breaks

DNA double-strand breaks are very dangerous DNA damages. Unrepaired DDSBs leads to apoptosis or senescence while a wrong DDSB repair can result in genomic instability and carcinogenesis. Four repair pathway allow to repair these DDSBs in eukaryotic cells: NHEJ, HR, SSA and alt-EJ (Ceccaldi, R. *et al.* 2016).

2.1.1. Non Homologous End-Joining

NHEJ, which is active at all stages of the cell cycle, consist in the religation of DNA ends whithout the help of a homologous template. It is composed of four steps: (1) DNA end recognition and assembly and stabilization of the NHEJ complex at the DDSB, (2) Bridging of the DNA ends and end stability, (3) DNA end processing and ligation of the broken ends and (4) dissolution of the NHEJ complex (Davis, A.J. *et al.* 2013).

To begin, the DDSB is recognized by the Ku heterodimer which binds at the lesion (Figure 3). The Ku heterodimer is composed of Ku70 and Ku80 subunits which are encoded by the XRCC5 (X-ray repair cross-complementing) and XRCC6 genes, respectively (Davis, A.J. *et al.* 2013). Crystallographic analyses have shown that the Ku heterodimer forms a ring-shaped structure around the DNA helix and allows the heterodimer to slide on the end of the DNA. These studies also have highlighted that Ku70/80 interacts with the sugar backbone of DNA, not with the bases, which gives it the ability to bind to DNA in a sequence independent manner (Walker, J.R. *et al.* 2001).

The Ku heterodimer bound to the DNA damage site acts as a scaffold to recruit the others NHEJ factors, including DNA-PKcs, Xray cross complementing protein 4 (XRCC4), DNA Ligase IV, XRCC4-like factor (XLF) and Aprataxin-and-PNK-like factor (APLF) (Figure 3) (Davis, A.J. *et al.* 2013). DNA-PKcs belong to the phosphatidylinositol-3 (PI-3) kinase-like kinase (PIKK) family. Their C-terminal and N-terminal domains form a pincer-shaped structure that probably allows the DNA-PKcs to bind to the dsDNA (Sibanda, B.L. *et al.* 2010). Ku70/80 makes numerous contacts with the N-terminal and C-terminal region of DNA-PKcs and these interactions lead to the translocation of the Ku heterodimer inward on the DNA strand (Davis, A.J. *et al.* 2013). XRCC4-DNA Ligase IV complex are also recruited to the DNA ends and the interaction between XRCC4 and Ku70/80 seems to recruit the processing enzyme at the DDSB for the DNA ends processing, the penultimate step of NHEJ (Mari, P.-O. *et al.* 2006, Nick McElhinny, S.A. *et al.* 1999).

The next step promotes bridging of the DNA end and their stabilization. This stabilization is essential to protect them from non-specific repair that could lead to chromosomal aberrations. The Ku heterodimer and XRCC4-XLF protect and stabilize them. DNA-PKcs are also at the extremities and allow the formation of a synaptic complex that contain the two pieces of DNA ends (Davis, A.J. *et al.* 2013).

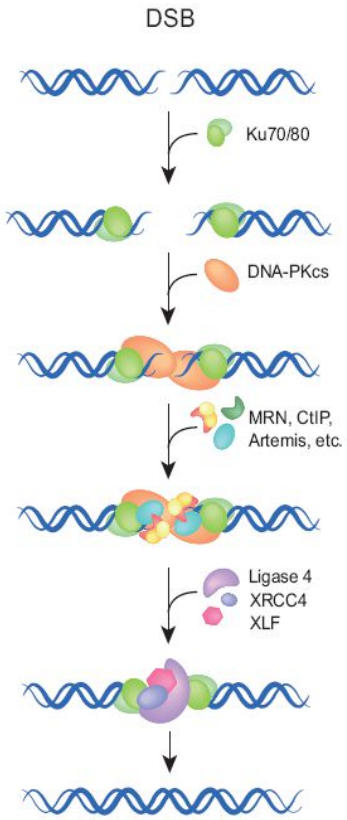

Figure 3. Repair of DDSB by Non-Homologous End Joining. From the Translational Cancer Research, Dueva, R. and Iliakis, G., 2013.

The ends of the DNA must then be processed before proceeding to the last step, which is the ligation of the ends. Depending on the type of breakage, the ends can be cut, the gaps can be filled and the terminal blocking groups can be removed. This makes it possible to make the ends liable. Different DNA end processing enzymes are available to make these modifications including Artemis, PNKP, APLF, Polymerases μ and λ, Werner (WRN), aprataxin and Ku. Artemis, WRN, and APLF perform the resection of DNA ends while the family of X polymerases, including DNA polymerases μ and λ, are necessary when DNA gaps need to be filled. PNKP and Aprataxin remove the blocking end groups like nonligatable 5′ hydroxyls or 3′ phosphates.

Finally, when DNA ends have been processed, they are ligated by DNA Ligase IV. This enzyme forms a complex with XRCC4, the latter allowing stabilization and stimulation of the activity of DNA Ligase IV. Ligase IV promotes the ligation of incompatible DNA ends but also the ligation across a gap.

Once the break is repaired, the NHEJ complex need to dissolve from the DNA. Some studies on Xenopus egg extracts showed the Ku80 is polyubiquitylated by Skp1-Cul1-Fbxl12 resulting in its dissociation from DNA and its degradation by the proteasome. Studies on human cells showed that Ku dissociation from DNA could by dependent on the E3 ubiquitin ligase RNF8. The phosphorylation status of DNA-PKcs is also important for the dissociation since autophosphorylation of DNA-PKcs leads to a conformational change and release from DNA (Davis, A.J. *et al.* 2013, Moore, J.K. *et al.* 1996).

2.1.2. Homologous recombination

As indicated by the name, a homologous template is used in homologous recombination to repair the DDSB. This pathway is composed of three steps: the presynapsis, the synapsis and the postsynapsis. HR is mainly effective during the S/G2 phase of the cell cycle, when NHEJ

can also occur. There is thus a choice to make between the two pathways (Jasin, M. *et al.* 2013).

The presynaptis phase begins when the nuclear enzyme PARP1 recognizes DDSB. It is rapidly activated and signals the DNA lesion by attaching ADP-ribose units to chromatin-associated proteins. It promotes the recruitment of Mre11 and Nbs1, two components of the MRN complex, and CtIP (Haince, J.-F. *et al.* 2008). Then the chromosome lesion undergoes nucleolytic resection to 3' OH ends. So the 5' ends are cut off while maintaining relatively stable 3 'ends (Figure 4). The MRN complex, composed by Mre11, Rad50 and Nbs1, and the CtIP protein are responsible for this resection. BRCA1 colocalizes with the MRN complex and CtIP and is also important since it cooperates with the MRN nuclease to catalyze resection (Jasin, M. *et al.* 2013, Prakash, R. *et al.* 2015).

The single-strand DNA (ssDNA) formed by this resection is covered by the replication protein A (RPA). However, Rad51, a member of the RecA-like NTPases, has to bond to ssDNA to continue HR. A mediator, the Breast Cancer 2 (BRCA2) protein, binds to Rad51 through its BRC repeats helping the Rad51 recombinase to bind to ssDNA (Jasin, M. *et al.* 2013, Prakash, R. *et al.* 2015). Rad51 forms right-handed filament around DNA. Those are stretchable, which is essential for a fast and efficient homology search (Jasin, M. *et al.* 2013, San Filippo, J. *et al.* 2008, Yu, X. *et al.* 2001).

During the synapsis, Rad51 filaments invade the homologous template allowing a connection between the invading DNA substrate and its homologous template. This leads to formation of a heteroduplex double-strand DNA called D-loop (Ferguson, D.O. *et al.* 1996, Jasin, M. *et al.* 2013).

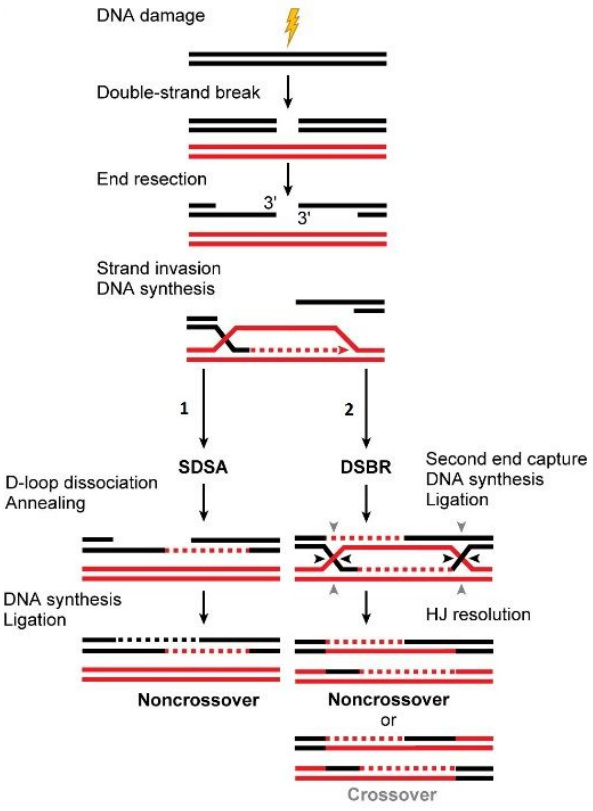

Figure 4. Repair of DDSB by Homologous Recombination (HR) using homologous template. The repair of the break can be done by different HR pathway including (1) Synthesis-Dependant Strand Annealing (SDSA) and (2) Double-Strand Break Repair (DSBR). Adapted from the Annual Review of Biochemistry, San Filippo, J., 2008.

The post-synaptic phase consists in the synthesis of DNA within the D-loop using the homologous template, while Rad51 dissociates from DNA (Figure 4). After launching DNA synthesis, three paths can be done and lead to a cross-over (CO) or not (NCO). A cross-over is an exchange between the DNA molecule initially broken and the template DNA. The Synthesis-Dependant Strand Annealing (SDSA) pathway includes the dismantling of D-loop, binding of the two extremity of DNA and filling of the single-strand DNA by DNA synthesis. Double-Strand Break Repair (DSBR) pathway is formation of two heteroduplex having two Holliday junctions (Schwacha, A. et al. 1995). Specific enzymes cleave the junctions and the resolution lead to formation of CO or NCO. However, the junction can be dissolved by the Sgs1–Top1–Rmi1 complex leading to only NCO. For Break Induced Replication (BIR) pathway, DNA synthesis begin at the D-loop, transformed into a replication fork, to the end of DNA. This occurs for example at the breaking level of a replication fork (Jasin, M. *et al.* 2013, San Filippo, J. *et al.* 2008).

2.1.3. Alternative End Joining

The alt-EJ repair pathway or Microhomology-Mediated EJ pathway (MMEJ) is the second pathway beginning by end resection that generates 3' ssDNA. Instead of using a homologous template from the sister chromatid, this pathway relies on microhomologies around the site of DDSB. This pathway is mutagenic because there are deletion and insertion of sequences between the microhomologies at the repair site. It is composed of 5 steps: end resection, DNA end bridging and aliment, removal of non-homologous 3' tail, DNA gap filling and ligation.

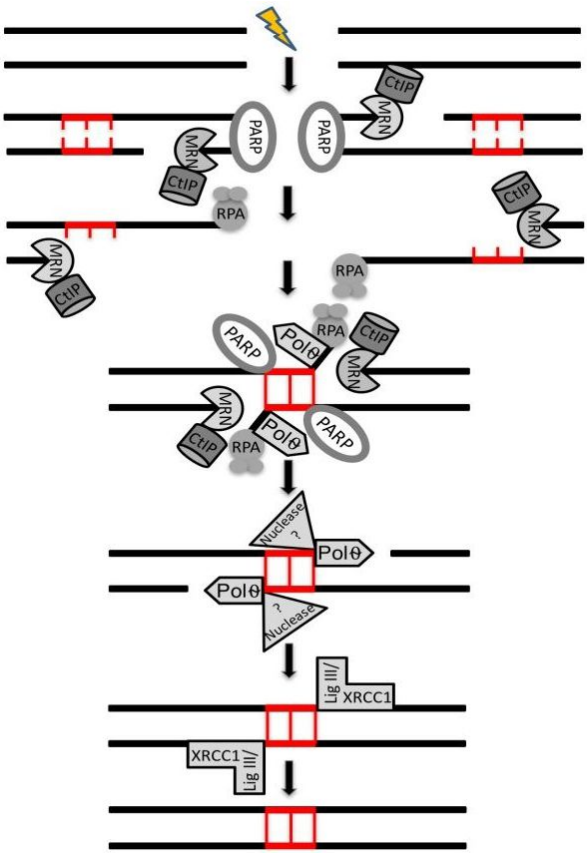

Figure 5. Repair of DDSB by two complementary sequences (in red) through Microhomology-Mediated End-Joining Pathway. From the Journal of Biological Chemistry, Sallmyr, A. and Tomkinson, A. E., 2018.

First, as in HR, PARP1 binds to DDSB, recruits the MRN complex and CtIP needed for DNA end resection and ssDNAs resulting from resection are covered by RPA (Figure 5). When microhomologies are present on the ssDNAs, they can anneal, bridging the two ends of DNA. The MRN complex has an end-bridging activity, allowing it to engage two DNA ends, and align the DNA ends. Pol θ is also a key factor in end alignment. Pol has an N-terminal helicase-like domain, a central unstructured region and a C-terminal polymerase domain. It is involved in end bridging because of its ability to aligning microhomologies. Then the non-complementary 3' tails are removed before gap-filling DNA synthesis. The specific DNA polymerase allowing the gap filling include Pol θ. Once DNA ends are ligatable, the DNA ligase III/XRCC1 complex ligate the DNA ends (Sallmyr, A. *et al.* 2018).

2.1.4. Single Strand Annealing

SSA is quite similar to a-EJ, except it involves complementary sequence of over 25 nucleotides in length. It is relatively mutagenic because the repeats lead to large deletion rearrangement between the repeats. The SSA pathway is composed of three steps: end resection, annealing synapsis and end processing ligation.

After DNA resection and RPA coating, an additional exonuclease, either Exo1 or DNA2, extend the resection to give access to the complementary regions (Figure 6). Rad52 interacts with RPA-coated singles strands and allows annealing of the flanking repeats due to its robust single annealing activity. In addition, the non-complementary 3' tails are removed by ERCC1, which is associate with XPF to form a protein complex having a nuclease activity. Finally DNA gap are filled by DNA polymerases and the two strands are ligate (Bhargava, R. *et al.* 2016).

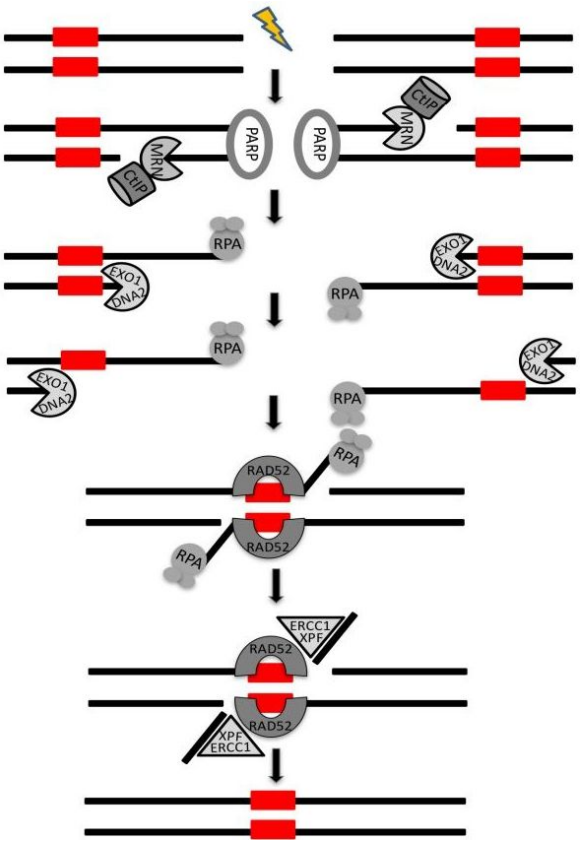

Figure 6. Repair of DDSB flanked by two complementary sequences (red boxes) through Single-Strand Annealing (SSA) pathway. From the Journal of Biological Chemistry, Sallmyr, A. and Tomkinson, A. E., 2018.

2.2. Translesion synthesis

DNA damages, such as pyrimidine dimers caused by UV rays, that are not repaired can, among other things, lead to replication fork arrest. To counteract this replication fork stalling, an important DNA-damage tolerance pathway called Trans-lesion synthesis (TLS) can be used. It requires a switch to specialized translesion synthesis DNA polymerases to incorporate nucleotides opposite and beyond the DNA lesion. This pathway is error-prone due to the lower fidelity and lack of proofreading activity of the TLS polymerases (Zhao, L. *et al.* 2017).

The TLS pathway begins when the replication fork faces a breakage such as UV-induced pyrimidine dimers. Replicative polymerases (Pol ε and Pol δ) are blocked by this damage but the CMG helicase (composed of CDC45 protein, MCM2-7 complex and the GINS complex) continues to unwind the DNA. The decoupling of helicase and polymerases leads to an exposure of ssDNA and the RPA protein is directly bound to it. Then the proliferating cell nuclear antigen (PCNA) binds to stalled fork, follow by the RAD6-RAD18 complex (Figure 7). These complexes induce mono-ubiquitination of PCNA at lysine 164 (K164) (Tsuji, Y. *et al.* 2008).

Monoubiquitinated PCNA serves as an interacting platform for Y-family TLS polymerases via their ubiquitin-binding domains (Hoege, C. et al. 2002, Zhao, L. et al. 2017). The TLS polymerases Polη have more affinity for monoubiquitinated PCNA than the normal Polδ or Pol ε, which explains the polymerase switch. Polη allow the insertion of bases opposite damaged nucleotides (Kannouche, P.L. *et al.* 2004, Masuda, Y. *et al.* 2010, Zhao, L. *et al.* 2017).

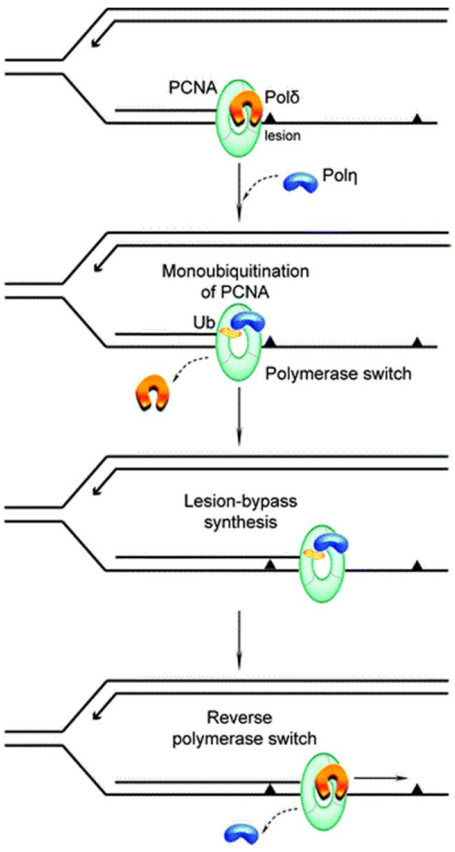

Figure 7. Translesion Synthesis pathway. This DNA damage tolerance pathway recruits specialized low fidelity TLS polymerase to bypass the lesions for repair. Adapted from Cell Biochemistry and Biophysics, Chen, J. et al., 2011.

3. DNA damage checkpoint

The DNA damage repair (DDR) pathways and the complex network of cell-cycle checkpoint proteins are activated following DNA damages. These cell-cycle checkpoint proteins are essential to block the cycle and allow time to repair DNA lesion properly. Even if the nature of the stress differs, the checkpoints activation pathway share similarities (Scott, S.P. *et al.* 2006, Shaltiel, I.A. *et al.* 2015).

The signaling proteins that initiates the DNA damage cell-cycle checkpoints are the protein kinases of the Phosphatidylinositol 3-kinase-related kinases family; ATM (Ataxia Telangiectasia Mutated), ATR (Rad3 related) and DNA-PK (DNA-dependent protein kinase). They recognize DNA damages and are activated at DNA damage sites. ATM is activated preferentially by DDSB while ATR is activated by a broad spectrum of damages including stalled replication forks and lesions induced by UV. ATM is also activated by MRN complex which rapidly bind to DDBS (Maréchal, A. *et al.* 2013, Scott, S.P. *et al.* 2006).

The activation of ATM or ATR leads to phosphorylation of a large number of substrates involved in DNA repair, cell-cycle arrest, apoptosis, etc., such as tumor protein p53, Chk1 and Chk2, involved in cell-cycle checkpoint, and histone H2AX (Figure 8) (Maréchal, A. *et al.* 2013). Upon DNA damage and other cellular stress, p53 is phosphorylated on ser15 by ATM, ATR or DNA-PK. This allows its dissociation from its repressor MDM2. Once activated, p53 acts as a transcription factor. It controls cell cycle, allowing damage repair, or induces senescence and apoptosis in response to cellular stresses (Kruse, J.-P. *et al.* 2009, Marchenko, N.D. *et al.* 2000, Mercer, W.E. *et al.* 1984). Histone H2AX is a key factor in DNA damage repair process. It is a variant of the H2A protein family which contribute to the histone octamer in nucleosomes. In presence of a DNA damage, H2AX is recruited to damage sites and phosphorylated on the 139th serine residue, predominantly phosphorylated by ATM. The phosphorylated form of H2AX called γ-H2AX allows the recruitment of DNA repair protein to damage site (Burma, S. *et al.* 2001, KUO, L.J. *et al.* 2008).

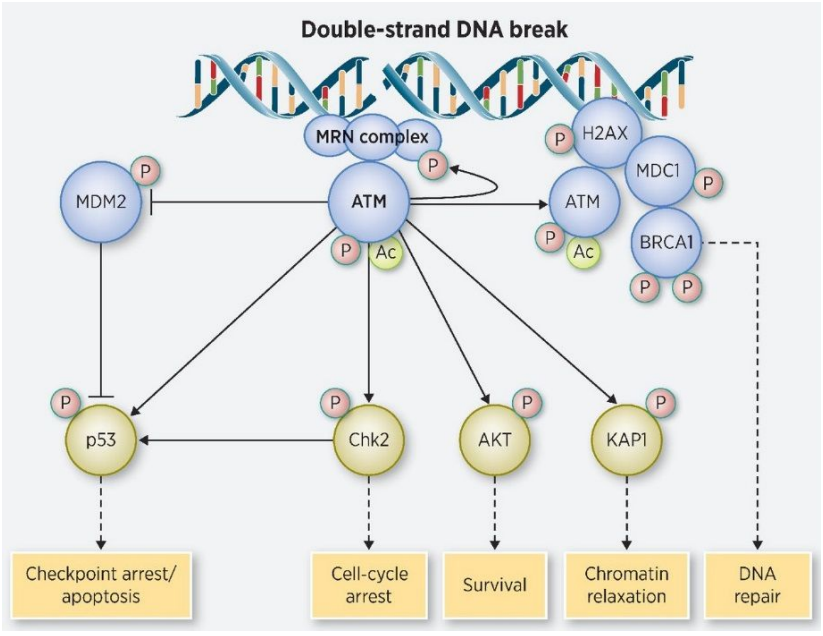

Figure 8. Regulation of DNA damage response by ATM. From AACR Journal, Choi, M., 2016.

4. MAGEs proteins

During the 1990s, the first Melanoma-associated Antigen Gene (MAGE) proteins have been identified as precursors of antigenic peptides presented by melanoma cells and recognized by cytolytic T lymphocytes *in vitro* (van der Bruggen, P. *et al.* 1991). The genes coding these proteins are now named *MAGEA1, A2 and A3*. Since then, other *MAGE* genes have been discovered, the MAGE family is now fully characterized and contains more than 50 genes that can be grouped in subfamilies (A, B, C, D, E, F, G, H, L) according to sequence similarities (Chen, Y.T. *et al.* 1994, Gaugler, B. *et al.* 1994, Li, X. *et al.* 2013). Furthermore, the sequencing of the genome of other eutherians showed that they also contain above 30 *MAGE* genes. Surprisingly, the non-placental mammals and non-mammalian vertebrates have a single *MAGE* gene in their genome. This is also the case of invertebrates where this *MAGE* gene is called *Nse3* (De Donato, M. *et al.* 2017, Zhao, Q. *et al.* 2012).
The evolution of the MAGE family is interesting because at first there was only a single-copy state of an ancestral gene that lasted until the emergence of eutherian mammals. Then *MAGE* genes were produced by retrotransposition. After that, there is extensive gene duplication within each subfamily and the sub-families were modified over time (Katsura, Y. *et al.* 2011). The sub-family MAGED1 is interesting because it is the closest to the ancestor *Nse3* gene. In addition, the MAGED sub-family has more than 91% identity in the coding sequences between human and dog, the species with further evolutionary distance. So this suggests that *MAGED* genes generated the other subfamilies by retroposition. MAGEG1 would be the MAGE having kept the initial Nse3 function (Chomez, P. *et al.* 2001, Taylor, E.M. *et al.* 2007).

MAGEs genes are categorized into two classes according to their expression profile. Type I MAGEs, composed of MAGEA, B and C subfamily members, are located on the X chromosome. They are only expressed in germ cells and are aberrantly express in various types of tumors. Type II MAGE, consisting of MAGED, E, F, G, H, L and Necdin genes, are not restricted to the X chromosome and are expressed in numerous tissues (Chomez, P. *et al.* 2001, Lucas, S. *et al.* 2000). All MAGE proteins share a common domain of about 170 amino acids called the MAGE homology domain (MHD) (Figure 9) (Chomez, P. et al. 2001). It is formed of two tandem winged-helix (WH) motifs named WH-A and WH-B. Each WH has a helix-tower-helix motif packed against a three-stranded antiparallel β-sheet "wing". WH-B also contains additional α helices (Doyle, J.M. *et al.* 2010, Newman, J.A. *et al.* 2016).

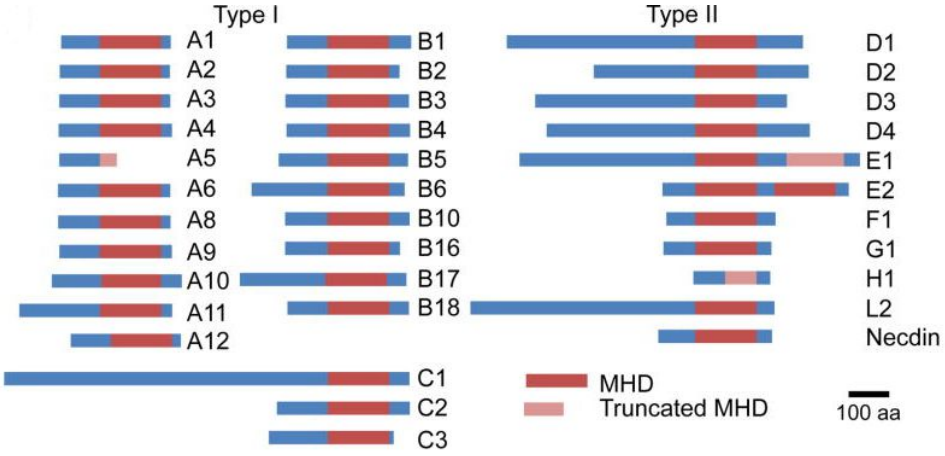

Figure 9. MHD domain. Adapted from Current Opinion in Cell Biology, Weon, J.L. and Potts, P.R., 2015.

Several studies have shown that MAGE proteins are involved in DNA damage repair, interact with the DNA damage sensor protein ATM, suppress the apoptosis and modulate the ubiquitination process.

Ubiquitination is an enzymatic post-translational modification regulating a large number of processes in eukaryotic cells including degradation of proteins by the proteasome, modification of their stabilization, their localization or their activity. This modification consists of the formation of bond between the C terminus glycin residue of ubiquitin (G76) and the ε-amino group of a substrate lysine residue. It is carried out in three steps by three enzymes: the ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2) and the ubiquitin-protein ligases (E3). First, an ubiquitin-adenylate intermediate is formed thanks to ATP. Then the intermediate reacts with E1 and forms a thiol ester with the carboxyl group of G76, allowing to activate the terminal C end of the ubiquitin for a nucleophilic attack. E2 transiently carries the activated ubiquitin molecule as a thiol ester and finally E3 transfers the activated ubiquitin from E2 to the substrate (or ubiquitin lysine residue) (Figure 10a). The E3 ligases are classified into four families, characterized by specific motifs: HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene), U-box (a modified RING motif without the full complement of Zn2+-binding ligands) and PHD-finger (plant homeodomain finger). Substrate recognition for ubiquitination is enabled by the presence and access to ubiquitination signals that are primary (or structural) sequence motifs in the substrate that are recognized by related E3 ligases. E3 are thus the determining enzymes for substrate specificity (Lorick, K.L. *et al.* 1999, Pickart, C.M. 2001).
The fate of the protein will depend on the number of ubiquitins it bears, whether it is an ubiquitin chain or not and the type of binding between lysine and glycine (Figure 10b). For example, proteins marked by a polyubiquitin chain in which successive ubiquitins are linked by K48-G76 isopeptide bonds are intended to be degraded by the proteasome while monoubiquitination can lead to endocytosis, gene expression, nuclear export or DNA repair (Hershko, A. *et al.* 1992, Pickart, C.M. 2001).

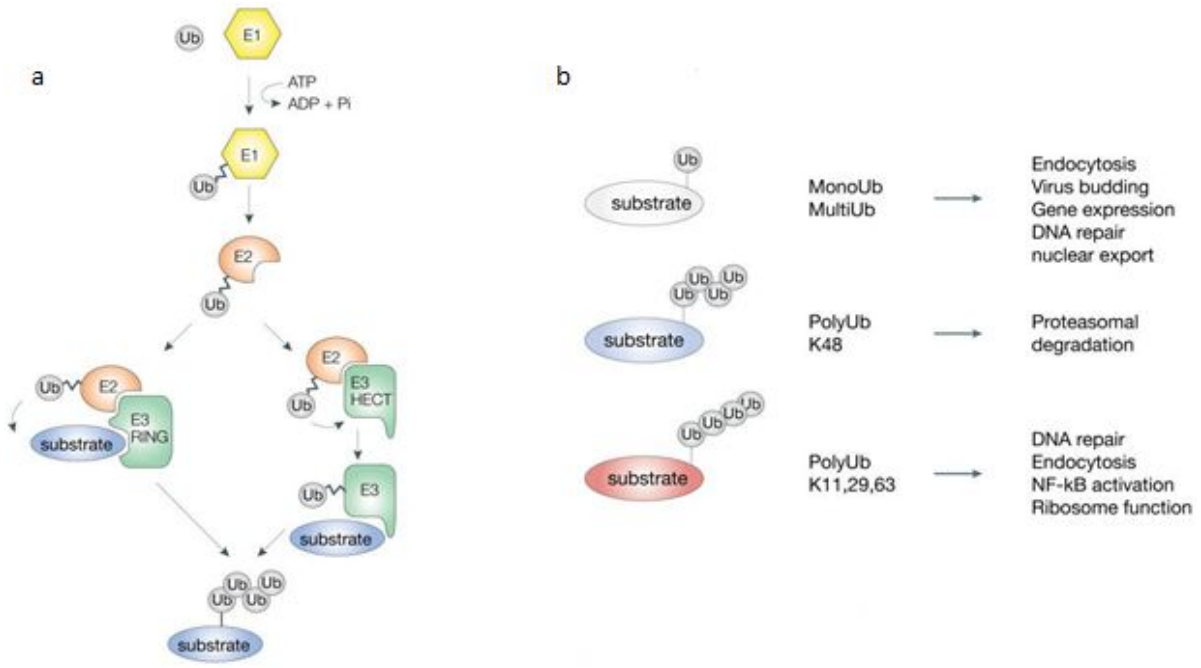

Figure 10. Ubiquitination process and the different fate according to the type of ubiquitination. (a) Schematic representation of the ubiquitination process requiring three types of enzyme: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes. (b) Representation of the different types of ubiquitination and the different associated fate. Adapted from Cell Division, Woelk, T. et al., 2007.

MAGE proteins interact with E3 ubiquitin ligases through their MHD. Each MAGE protein seems to bind specifically one particular E3 ligase but one E3 ligase can interact with different MAGE proteins. Several observations of the role of MAGE proteins in the ubiquitination of proteins in the context of cancer cells have been made (Doyle, J.M. *et al.* 2010).

For instance, MAGEA2 binds MDM2 (murine double minute 2), an E3 ubiquitin ligase which mediates ubiquitination of several substrates, including p53 and MDM4. There is a feedback loop in which p53 induces MDM2 expression leading to downregulation of p53 by MDM2. MDM4 is the partner of MDM2, being structurally similar to him, and is an inhibitor of p53 since it sterically blocks the interaction between p53 and the transcriptional machinery.
The interaction between MAGEA2 and MDM2 is mediated by the N-terminal p53-binding pocket and the E2-binding surface within the C-terminal RING structure of MDM2 (Chen, J. et al. 1993, Marcar, L. et al. 2015). Since the RING finger domain is required for homo / heterodimerization and E2 ligase interaction, MAGEA2 appears to be an inhibitor of MDM2 E3 ubiquitin ligase activity. MAGEA proteins can also disrupt the interaction between MDM2 and MDM4, inducing an increased level of MDM4 (Doyle, J.M. *et al.* 2010, Marcar, L. *et al.* 2015)
MAGEA3 and MAGEA6 interact with the E3 ubiquitin ligase TRIM28 (also known as KAP1), enhancing the ubiquitination and degradation of AMPKα1. AMPKα1 is a sub-unit of the AMP activated protein kinase (AMPK) which is a major sensor and regulator of cellular energy. Indeed, this hetero-trimeric kinase (α, β and γ subunits) is regulated by the cellular concentration of ATP, ADP and AMP and generally represses anabolic energy-consuming pathways while it promotes catabolic ATP-generation pathways. It is involved in cascades which lead to the phosphorylation of the autophagy-initiating kinase (Ulk1) on Ser317 and Ser777 and inhibition of mammalian target of rapamycin (mTOR), finally engaging autophagy (Doyle, J.M. et al. 2010, Kim, J. et al. 2011, Pineda, C.T. et al. 2015). The downregulation of AMPK deregulates the cellular metabolism since there is a loss of autophagy, which is favorable to tumorigenesis.
MAGEA3 and MAGEC2 interact with TRIM28 and enhance the degradation of FBP1 (Fructose-1,6-biphosphatase) (Doyle, J.M. et al. 2010, Jin, X. et al. 2017). FBP1 is a rate-limiting enzyme acting on gluconeogenesis and antagonizes glycolysis because it allows conversion of fructose-1,6-bisphosphate in fructose-6-phosphate. When FBP1 is degraded, there is an increased glucose uptake resulting in increased glycolysis. It thus promotes the Warburg effect and hepatocellular carcinoma (HCC) progression. This effect occurs when cancer cells tend to favor metabolism via aerobic glycolysis rather than the much more efficient oxidative phosphorylation pathway.
MAGEC2 enhances TRIM28-dependent ubiquitination and degradation of tumor suppressor p53, suppressing apoptosis and enhancing tumor survival (Doyle, J.M. et al. 2010).
Other type I and type II MAGE proteins seem to be involved in DNA damage repair.

4.1. Type I MAGE and DNA damage repair

MAGEA4 is a type I MAGE that can modulate the DNA-damage tolerance pathway of TLS. When cancer cells depleted of MAGEA4 are treated with genotoxic agent (CPT) or UV, they display a delayed resumption of DNA synthesis and S-phase arrest.
Studies on H1299 adenocarcinoma cells have shown that MAGEA4 interacts with the E3 ubiquitin ligase RAD18. The UV or CPT, genotoxins activating the distinct pathways of the RAD18 TLS and DSB repair effector, do not influence the presence of MAGEA4 in the RAD18 complex. Another finding is that MAGEA4 is ubiquitinated by RAD18. It has been shown that a high MAGEA4:RAD18 ratio resulted in a decrease in the mono-ubiquitination of PCNA in vitro because of substrate competition.
Furthermore, it was shown that MAGEA4 promotes RAD18 stabilization and that MAGEA4 depletion led to a decrease of PCNA mono-ubiquitination. This is the di-leucine motif of

MAGEA4 which, by interacting directly with the RAD6-binding motif of RAD18, stabilizes it. The interaction between MAGEA4 and RAD18 is therefore unique compared to other MAGEs since MAGEA4 does not promote the E3 ligase activity of RAD18 but allows its stabilization. In addition, through this stabilization, MAGEA4 promotes the mono-ubiquitination of PCNA. This therefore makes it possible to strengthen TLS (Gao, Y. *et al.* 2016).

MAGEC2, another type I MAGE, promotes DDR through the DNA damage sensor protein ATM. It binds to the E3 ubiquitin ligase TRIM28, facilitating its phosphorylation on Ser-824 by ATM under cellular stress conditions (Bhatia, N. *et al.* 2012). This phosphorylation is an early event in DNA repair and is required for chromatin relaxation allowing access of DNA repair proteins. TRIM28, like H2AX, is organized into foci that surround DNA lesions, promoting DDR. Once DNA is repaired, KAP1-Ser824 is dephosphorylated, allowing chromatin recondensation (White, D.E. *et al.* 2006, White, D.E. *et al.* 2011). Studies have shown that MAGEC2 promotes survival of melanoma cell both in vitro and in vivo and that its downregulation leads to apoptosis (Bhatia, N. *et al.* 2012).

4.2. Type II MAGE and DNA damage repair

MAGEG1, a type II MAGE, is part of the Structural Maintenance of Chromosomes (SMC) 5-6 complex. The SMC5-6 complex of yeast is composed of Smc5, Smc6, both containing Walker A and Walker B motifs at N- and C-terminal globular domains, and six non-SMC elements (Nse1– 6) (Diaz, M. *et al.* 2018). MAGEG1 is the human orthologue of Nse3 (Taylor, E.M. et al. 2007). The SMC5-6 complex has an essential role in DNA repair and regulates chromosome dynamics, structure and function in eukaryotes. It promotes homologous recombination between sister chromatids after double-strand break (De Piccoli, G. *et al.* 2006). SMC5-6 is recruited at the DSB in yeast and in humans and would allow recruitment and/or maintenance of the cohesin complex at the DSB (Potts, P.R. *et al.* 2006). SMC5-6 also promotes collapsed replication forks repair. It maintains forks in recombination-competent configurations and helps the resolution or prevents the formation of certain recombination intermediates (Ampatzidou, E. *et al.* 2006).
In this complex, MAGEG1 interacts with Nse1, a RING finger protein with E3 ubiquitin ligase activity. The role of the interaction between MAGEG1 and Nse1 is not fully understood but it would be involved in DNA repair (Hudson, J.J.R. *et al.* 2011)
In Drosophila, DNA damage repair by HR requires both ATM and ATR. Studies have been carried out to find proteins involved in caffeine resistance. A screen revealed that under normal growth conditions, *Smc5*, *Smc6* and *MAGE/Nse3* were not essential but were essential for resistance to caffeine exposure throughout development. MAGE and Smc6 interacted with ATM and ATR required for DDR in a caffeine-dependent manner, allowing the genome stability. Others observations have shown that an improper Rad51 activity (enzyme involved in DNA repair) was the cause of the caffeine sensitivity of the SMC5/6 and MAGE mutants. These studies also looked if the drosophila Smc5/6 promotes resistance to others agents inducing genotoxic stress. MAGE, Smc6 and Smc5 mutants were exposed to CPT, ionizing radiation, hydroxyurea, which inhibits of DNA replication, and methyl methanesulfonate, an alkylating agent. The three mutants were hypersensitive to all these agents. These observations highlighted a role of Smc5/6 and MAGE in cell response and resistance to genotoxic stress (Li, X. *et al.* 2013).
In humans, missense mutations of MAGEG1 are the cause of a chromosome breakage syndrome called lung disease immunodeficiency and chromosome breakage syndrome (LICS). Those mutations disturb the stability of Smc5/6 and HR. The syndrome is characterized by an immune-deficiency, failure to thrive, absent (or mild) dysmorphic features and severe pediatric pulmonary disease (van der Crabben, Saskia N *et al.* 2016).

A role for MAGED1 (also known as NRAGE), another type II MAGE, in DDR was first pinpointed by the observation that phosphorylation level of γ-H2AX, a DDSB biomarker, increases when MAGED1 is knockdown (Yang, Q. *et al.* 2016).
These studies then wanted to know in which repair pathway MAGED1 is involved. It was shown that neither downregulation nor overexpression of MAGED1 affect the NHEJ pathway. However, when MAGED1 is downregulated, HR is less efficient while it was increased 10 times when MAGED1 is overexpressed. MAGED1-deficient EC cells also display much more DSB and were much more sensitive to irradiation, cisplatin, a chemotherapeutic agent inducing DNA damage by crosslinking with the purine bases on DNA, and etoposide, a topoisomerase-II poison. MAGED1 thus protects from a wide range of DNA-damaging agents promoting chemoresistance of EC cells.
Other cell types, including mouse embryonic fibroblast cells, were then used to verify that the role of MAGED1 in DDR was not specific to EC cells. Immunofluorescence showed that MAGED1 delocalized into nucleus from cytoplasm after exposure to UV and partially colocalized with γ-H2AX (Yang, Q. *et al.* 2016).
The E3 ubiquitin ligase RNF8 and the BRCA1-associated RING domain protein 1 (BARD1), two factors of DDR, were much more polyubiquitinated in MAGED1-deficient cells. This suggests that MAGED1 promotes the stabilization of those proteins via the ubiquitin-proteasome pathway. It was shown that MAGED1 directly interact with the RING domain of both factors. RNF8 interacts with the DNA polymerase III subunits domain of MAGED1 while BARD1 interacts with the MHD-IRD of MAGED1. Simultaneously, RNF8 interact the Ankyrin-BRCT domain of BARD1 through its RING domain. MAGED1 is thus a chaperones that form the ternary complex MAGED1-RNF8-BARD1.
Overall, MAGED1 seems to conserve the genomic stability and helps cells to survive after DDSB after the exposure to different DNA-damaging agents (Yang, Q. *et al.* 2016)

Finally, MAGED2 is a poorly characterized type II MAGE protein which is highly conserved in mammals. It has been shown recently that it delocalizes after genotoxic stress and recently as a protein that modulates DDR (Pirlot, C. *et al.* 2016, Trussart, C. *et al.* 2018).
Previous studies have shown that MAGED2 expression is important because it inhibits TRAIL-induced apoptosis. This protection against TRAIL-induced apoptosis could allow melanoma cells to escape immune cell-induced apoptosis (Tseng, H.Y. *et al.* 2012). Studies have shown that mutations of MAGED2 causes antenatal Bartter's syndrome, which is characterized by polyhydramnios, prematurity, fetal polyuria and postnatal polyuria with persistent loss of renal salt (Laghmani, K. *et al.* 2016). It has also been shown that under normal condition MAGED2 is localized in the cytoplasm, nucleus and nucleol. Delocalisation of MAGED2 in the nucleoplasm is observed during the interphase and after a treatment with camptothecin or other DNA damaging drugs (Pirlot, C. *et al.* 2016).
MAGED2 overexpression or downregulation modulate p21 expression, a DNA damage check point kinase inhibitor. Depletion of MAGED2 in cells treated with CPT leads to the reduction of p21 protein and mRNA levels. It was shown that this reduction is independent of p53 activation (Trussart, C. *et al.* 2018).
The depletion of MAGED2 also affect both ATM and ATR kinases. ATM activity was reduced in MAGED2-depleted cells following CPT treatment. On the other hand, ATR activity was higher in MAGED2-depleted cells.
Thus, MAGED2 affects the cell cycle by impacting different regulators but also modulates DDR through the ATM/CHK2 and ATR/CHK1 branches of the DDR (Trussart, C. *et al.* 2018).
We have set up an assay to analyze the involvement of MAGED2 in DDR, developed by Bindra R.S. et al. which allows to measure NHEJ and HR repair activity in mammalian cells.

II. AIMS OF THE WORK

The objective of this study is to investigate if MAGEA and/or MAGED2 play a role in genotoxic stress resistance to camptothecin and DNA damage response in MelJuso cells and if its role is p53-dependent.

III. MATERIALS AND METHODS

Cell culture.

The human melanoma cell line MelJuso and the p53-knockout MelJuso cells were grown in Dulbecco's Modified Eagle's medium (DMEM) High Glucose (Biowest L0104) supplemented with 10% fetal bovine serum (Sigma F7524) and 1% penicillin-streptomycin solution (Biowest L0022). Cells were splitted twice a week and incubated at 37°C and 5% CO_2.

SiRNAs transfection.

Cells were plated in 25 cm^2 culture flask (1 million cells/flask) or 10 cm^2 well (250 000 cells/well). The transfection was performed 24 hours after cells seeding and when the cells were at 50% confluency. siRNAs were transfected using JetPRIME reagent (Polyplus transfection 114-07) according to the manufacturer's protocol with following siRNA: siCT (Negative Control siRNA Eurogentec SR-CL000-005), siMAGEA (MAGEA siRNA Eurogentec 5'-AACCAGCUAUGUGAAAGUC-3') and siMAGED2 (MAGED2 siRNA SMARTpool Dharmacon M-017284-00-0010). Transfected cells were incubated 48 hours at 37°C and 5% CO_2.

Camptothecin Treatment.

$2{,}5x10^5$ cells were plated in 10 cm^2 well and were transfected twenty-four hours later with siCT or siMAGEA using JetPRIME reagent and buffer. Forty-eight hours after transfection with siRNAs, MelJuso cells were treated with 5 µM of camptothecin (Sigma C9911) during 0 min, 30 min, 1h, 2h, 4h, 6h, 8h, or 24h. Proteins were extracted from cells with a lysis buffer to conserve the phosphorylation of γ-H2AX [1:2 Tampon 2X (8 M Tris, 25 mM EDTA (Ethylenediamine tetraacetic acid disodium salt dehydrate), 30 M Potassium chloride, 1% Triton® X 100, supplemented with cOmplete Protease Inhibitor Cocktail (Sigma 11697498001) and PhosSTOP (Sigma 04906845001)]. Cells were then centrifuged at 14000 rpm at 4°C for 15 min and the supernatant was stored for western blot.

For the recovery analysis, cells were treated after transfection with 5 µM of camptothecin during 4 hours. Then the drug-containing medium was removed and replaced with drug-free medium. The proteins were extracted with the aforementioned lysis buffer at different time points.

MTS assay.

MelJuso cells or clone E1 transfected with siCT or siMAGEA from 25 cm^2 culture flask were plate in 0,33 cm^2 well (15 000 cells/well). Twenty-four hours after plating, cells were exposed to different concentrations of camptothecin (0 µM, 0,5 µM, 1 µM, 2,5 µM, 5 µM, 10 µM, 25 µM or 50 µM) and then incubated for 24 hours at 37°C and 5% CO_2. MTS assay was performed using the CellTiter 96® AQueous One Solution Reagent (Promega G5421) according to the manufacturer instructions.

Immunoblotting.

Cells were trypsinized and then centrifuged at 4°C and 1200 rpm for 5 minutes. The cells pellets were lysed with NP40 lysis buffer (50 mM Tris, 150 mM Sodium chloride, NP-40 and 1:100 Halt Protease Inhibitor Cocktail (Thermo Scientific 78437) and incubated on ice for 10

minutes. Samples were centrifuged at 4°C and 14000 rpm for 10 minutes. Concentrations were determined with Pierce® 660 nm Protein Assay Reagent (ThermoFisher® 22660).
For γ-H2AX analyses, cells were lysed in the appropriate buffer (see "camptothecin treatment")

Proteins were separated by electrophoresis on SDS-polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes (Thermo Scientific 88018). The membranes were blocked (TBS-T/Tween 0,1% + 5% non-fat dry milk (Regilait)) for 15 minutes and washed three times for 5 minutes with TBS-T/Tween 0,1%. Membranes were then incubated overnight at 4°C with appropriate primary antibodies: MAGEA (Invitrogen 35-6300, 1:500), MAGED2 (Invitrogen 1145, 1:1000), PARP and cleaved PARP (Cell Signaling 9532, 1:1000), γ-H2AX (Cell Signaling 2577, 1:1000) and GAPDH (Sigma G8795, 1:10 000). Membranes were washed three times for 5 minutes and incubated for one hour at room temperature with the secondary horseradish peroxidase (HRP) anti-rabbit (Cell signaling 7074S, 1:2000) or anti-mouse antibody (Cell signaling 7076S, 1:2000). The proteins were detected with the Western Lightning Chemiluminescent reagent *Plus* (PerkinElmer NEL104001EA).

Cellular DNA extraction and PCR.

Cells were trypsinized and centrifuged at 1200 rpm for 5 minutes. Cells pellets were resuspended in the lysis buffer [200 mM NaCl, 100 mM Tris, 5 mM EDTA, 0,2% Sodium dodecyl sulfate (SDS) and 0,4 mg/mL of Proteinase K (Thermo Scientific EO0491)] and incubated overnight at 37°C. The lysate was then centrifuged at 14000 rpm for 10 minutes. The supernatant was kept for ethanol precipitation of DNA.

Electrophoresis was performes on 0.7% agarose gel to evaluate DNA concentrations. GoTaq green master mix (Promega M7423) was used for PCR with specific following oligonucleotides sequences: P53_screen_ex2_F 5'-CTCAGACACTGGCATGGTGT (n°1), P53_screen_ex2-9_R 5'-GGAGCCATTGTCTTTGAGGC (n°2) and P53_indel_R 5'-CATTGCTTGGGACGGCAAG (n°3). Primers 1 and 2 used together amplified the TP53 gene (exon 2 to exon 9) and primers 1 and 3 used together amplified the exon 2 region. The PCR conditions were 94°C for 3 *minutes,* 30 cycles of 94°C for 30 second, 57°C for 30 second, 72°C for 1 minute with a final extension step of 72°C for 10 minutes. PCR products were visualized by electrophoresis.

Sequencing by TA-cloning.

PCR products were cloned in the PCR 2.1 plasmid using the TA Cloning™ Kit. Ligation products were electroporated in 5-alpha electro-competent *E. coli* which were grown on antibiotic (Ampicillin) containing agar plates.

Several colonies for each condition were screened by PCR [using M13_fwd 5'-GTAAAACGACGGCCAGT (F) and M13_rev 5'-CAGGAAACAGCTATGAC (R)]. PCR conditions were 94°C for 3 *minutes,* 30 cycles of 94°C for 30 second, 57°C for 30 second and 72°C for 1 minute and 15 seconds, with a final elongation step of 72°C/10 minutes. Positive clones were cultured in LB medium overnight at 37°C for DNA extraction.

1.4 mL of culture was collected and centrifuged at 14000 rpm for 1 minutes. The supernatant was removed and 100 µL of GTE (50 mM glucose, 25mM Tris-Cl pH8, 10mM EDTA) solution was placed on the cells, vortexed and incubated for 5 minutes at RT. Then 150 µL of NaOH/SDS (0,2N NaOH, 1% SDS) solution was added and samples were incubated on ice during 5 minutes. After that 120 µL of Kac/Hac (12mL Kac 5M, 2,3 mL HA, 5,7 mL water) solution was added and the samples were mixed and then centrifuged for 5 minutes. The supernatant was transferred in 400 µL of phenol, vortexed and centrifuged for 5 minutes. The

aqueous phase was transferred in a new tube and DNA was isolated using EtOH precipitation. DNA concentrations were measured with a nanodrop. Samples were sequenced with M13 F and R.

RNA extraction and qPCR.

Cells from 10 cm^2 well (250 000 cells/well) were lysed in 2 mL of RoboZol RNA Extraction Reageant (VWR, N580-200ML), scrapped and placed at -80°C. Each sample was centrifuged at 13 000 rpm at 4°C for 10 minutes. The supernatant was transferred into a new tube and incubated at RT for 5 minutes. 200 µL of chloroform (VWR, UN1888) were added per mL of reagent, vortexed and incubated at RT for 15 minutes. Then it was centrifuged at 13 000 rpm at 4°C for 15 minutes. For the extraction of the RNA itself, the aqueous phase was recovered and 500 µL of isopropanol (VWR, 20839.366) per mL of reagent used above was added and vortexed. It was incubated for 5 to 10 minutes at RT and then centrifuged at 14 000 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet is washed with 1 mL of 75% EtOH (Fisher Scientific, E/0600DF/17). After centrifugation at 9000 rpm at 4°C for 10 minutes, the pellet was dried and resuspended in RNase Free water. Samples were dosed with nanodrop. Then, the RNA was converted into complementaryDNA using the GoScript™ Reverse Transcription Mix, Random Primer protocol (Promega 9PIA280) from 5 µg RNA according to manufacturer instructions.

IV. RESULTS

1. Assessment of the role of MAGEA in genotoxic stress resistance and DNA damage response in context of CPT treatment

A. Assessment of the impact of MAGE depletion on cell survival after camptothecin (CPT) treatment

As described above, CPT inhibits the topo-I and forms a stable ternary complex (CPT-DNA-topo-I). This complex prevents the DNA religation step and collides with the replication fork leading to DDSB and cytotoxicity. As some MAGEA have been described as being involved in DDR, we wanted to determine if MAGEA proteins protect cells from genotoxic stress. Viability assays were performed to determine if the depletion of MAGEA impacts resistance to CPT.

MelJuso cells were transfected with Negative Control siRNA (siCT) or a pool of siRNAs targeting MAGEA (siMAGEA). Those MAGEA siRNA were specific for the following MAGEA: MAGEA1, A2, A3, A4, A6, A7, A8, and A12. Depletion of MAGEA proteins was verified by western blot analysis using a pan-MAGEA antibody, which allows the detection of MAGEA1, A2, A3, A4, A6, A10 and A12 (Figure 11A).

The MTS assay showed a decreasing cell viability upon CPT treatment in siCT and siMAGE-A transfected cells up to the 2,5µM dose. Higher doses did not induce in higher loss of viability. MAGEA depleted cells were more sensitive to CPT from 2,5µM, with about 20% less surviving cells compared to the control cells. Thus, the downregulation of MAGE-A significantly reduces resistance to camptothecin treatment (p-value < 0,0001) and a significant difference is obtained for each concentrations tested from 2,5µM using the Bonferroni post-test (Figure 11B). This experiment was performed three times, the results being presented as a mean of three. A two-way ANOVA allowed to determine the statistical significance and Bonferroni post-test allowed to compare each average of each concentration two by two.

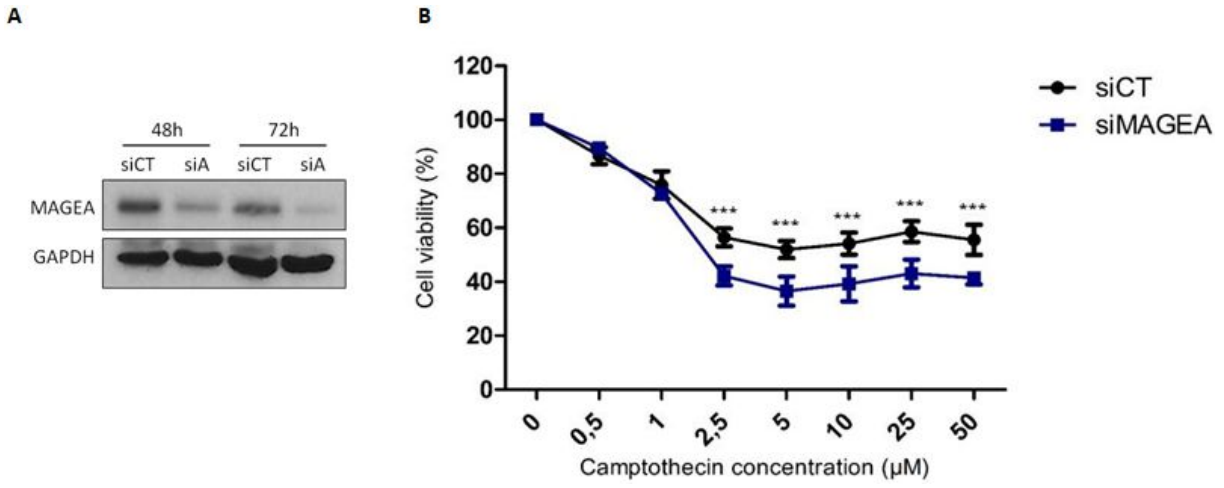

Figure 11. siRNA knockdown of MAGEA reduces the resistance of MelJuso cells to camptothecin treatment.
(A) Western blot showing MAGEA expression after 48h and 72h of transfection with a negative control siRNA (siCT) or a MAGEA siRNA (siA) in MelJuso cells. GAPDH was used as a loading control.
(B) MelJuso cells transfected with siCT or siMAGEA were treated during 24 hours with Camptothecin at the indicated concentrations. The cell viability was then measured by a MTS assay. Each point represents the mean of three independent experiments and the error bar represent the standard error. The data were analyzed with a two-way ANOVA and a Bonferroni post-test.

We wanted to determine if this decrease in cell viability after CPT treatment was dependent on p53 or not, as MAGEA proteins were previously shown to regulate p53 (Doyle, J.M. *et al.* 2010, Marcar, L. *et al.* 2010).

We decided to generate TP53-ko cells using the CRISPR/Cas9 technology, to be able to directly compare CPT resistance of those cells to our previous results on WT cells. Several clones were obtained that displayed a large deletion of TP53 on at least one allele. A western blot analysis allowed us to chose one clone. One p53-KO clone were transfected again transfected with Negative Control siRNA (siCT) or MAGEA siRNA (siMAGEA). This siRNA was different from the one used in the other experiments since it allowed to reduce the expression of the following MAGEA: MAGEA1, A2, A3, A4, A6, A10 and A12. Knockdown of MAGEA was confirmed by immunoblotting at 48 and 72 hour post-transfection (Figure 12A).

A decrease in cellular viability was still observed between the siCT and siMAGEA conditions in the p53-KO cells after CPT treatment. This decrease in cell viability was similar to the one observed above, with about 20% less viability in cells depleted in MAGEA. Again, the difference was significative (p-value < 0,0001) and a significant difference between each concentrations from 5µM was observed (Figure 12B). This experiment was performed three times, the results being presented as a mean of three. A two-way ANOVA allowed to determine the statistical significance and Bonferroni post-test allowed to compare each average of each concentration two by two.

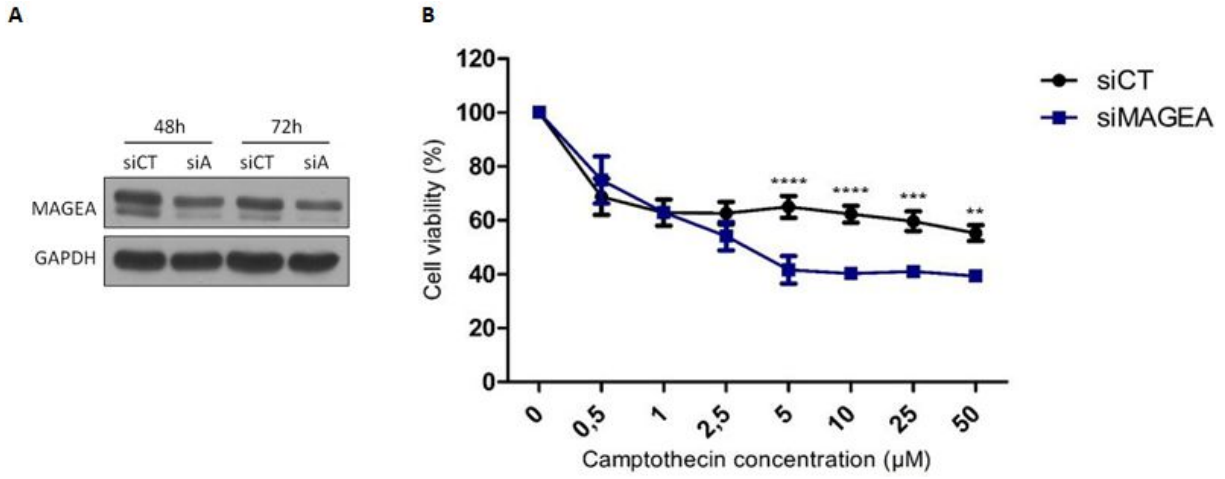

Figure 12. The role of MAGEA in genotoxic stress resistance to camptothecin is p53-independent.
(A) Western blot showing MAGEA expression after 48h and 72h of transfection with a negative control siRNA (siCT) or a MAGEA siRNA (siA) in p53-ko MelJuso cells. GAPDH was used as a loading control.
(B) p53-ko MelJuso cells transfected with siCT or siMAGEA were treated during 24 hours with Camptothecin at the indicated concentrations. The cell viability was then measured by a MTS assay. Each point represents the mean of three independent experiments and the error bar represent the standard error. The data were analyzed with a two-way ANOVA and a Bonferroni post-test.

B. γH2AX kinetic in MelJuso cells treated with camptothecin and depleted or not in MAGEA

We then wanted to know if the decreased in viability following CPT treatment in MAGEA-depleted cells could be explained by an impairment of DNA damage response or in DNA

repair. We chose to analyse the kinetic of the phosphorylation of H2AX, an early marker of DDSB.

We had performed a pre-test of cellular viability on MelJuso cells which allowed us to determine that at the 5µM concentration there was still 50% cellular viability and therefore that the CPT had induced enough damage for us to observe γ-H2AX. MelJuso cells depleted or not in MAGEA were treated with 5µM CPT for different times. A western blot analysis was performed to confirm the MAGE-A knockdown and to analyze the γ-H2AX kinetic. The experiment was performed three times.

Until 1 hour there was no signal for γ-H2AX either in the control cells or in the cells depleted for MAGEA. In the control cells the signal for γ-H2AX appeared at 2 hour and was quite weak. It remained low even at 4 hour and increases between 6 and 24 hour of treatment. In MAGEA-depleted p53-kO cells the signal for γ-H2AX also appeared at 2 hour but was much higher. The signal remained more intense at the other points. Again, analysis of MAGEA expression immunoblotting confirmed that MAGEA proteins were depleted in cells transfected with siMAGEA (Figure 13).

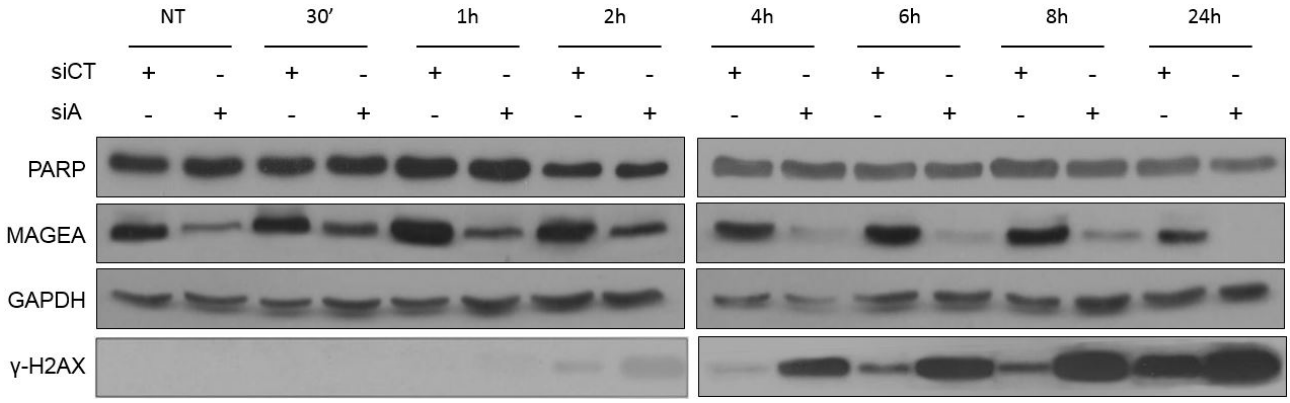

Figure 13. MAGE-A knockdown impact the kinetics of γ-H2AX in MelJuso cells.
MelJuso cells were transfected with a negative control siRNA (siCT) or a MAGEA siRNA (siA). Cells were exposed to 5 µM of Camptothecin during different times (30 min, 1h, 2h, 4h, 6h, 8h, and 24h) or not (NT). Then a western blot was carried out to analyze MAGEA and γ-H2AX expression where GAPDH was used as a loading control. The experiment was performed three times.

Again, we wanted to see if these results were impacted or not by the absence of p53 and therefore if the role of MAGEA in DDR and in genotoxic stress resistance is p53 dependent.

The same experiment was performed on our p53-KO MelJuso clone

We observed similar results to those above. The analysis of MAGEA expression was also done by immunoblotting and confirmed that MAGEA are depleted in cells transfected with siMAGEA. Overall, we observed no effect of p53 KO on the increase of histone H2AX phosphorylation following DNA damage in MAGEA-depleted cells (Figure 14).

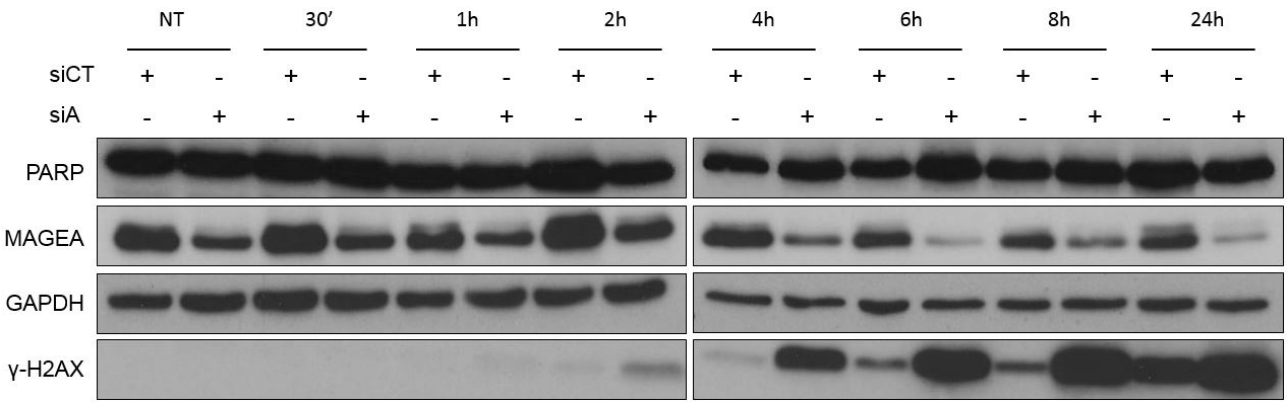

Figure 14. The role of MAGEA in genotoxic stress resistance to camptothecin is p53-independent.
p53-ko MelJuso cells were transfected with a negative control siRNA (siCT) or a MAGEA siRNA (siA). Cells were exposed to 5 µM of Camptothecin during different times (30 min, 1h, 2h, 4h, 6h, 8h, and 24h) or not (NT). Then a western blot was carried out to analyze MAGEA and γ-H2AX expression where GAPDH was used as a loading control. The experiment was performed three times.

C. Effect of depletion of MAGEA on the recovery of MelJuso cells after CPT treatment

We then wanted to observe the impact of MAGE depletion on the disappearance of γ-H2AX, indicative of efficient DNA repair.

MelJuso cells were transfected with siCT or siMAGEA and treated with 5 µM of camptothecin during 4 hours. We had chosen to expose the cells to the CPT for 4 hours because non-recovery experiment had shown us that after 4 hours of treatment there was a strong signal for γ-H2AX. After the treatment, the cells were released in a drug-free medium for different timings to allow them to recover from DNA damages induced by CPT. A western blot was performed to confirm the knockdown of MAGE-A to analyze γ-H2AX. The experiment was performed three times.

As expected on the basis of the non-recovery experiment, there was already a higher signal for γ-H2AX at time 0, the time at which the cells were released from the drug, in the cells depleted for MAGEA compared to the control cells. The signal stouts to decrease from 1h after release both in MAGEA-depleted and control cells. It nearly completely disappeared for the control cells 2h after release, while it persisted for MAGEA-depleted cells. 4 hours after release, however, no difference was observed between control and MAGEA-depleted cells. Western blot analysis confirmed that MAGEA are depleted in cells transfected with siMAGEA (Figure 15).

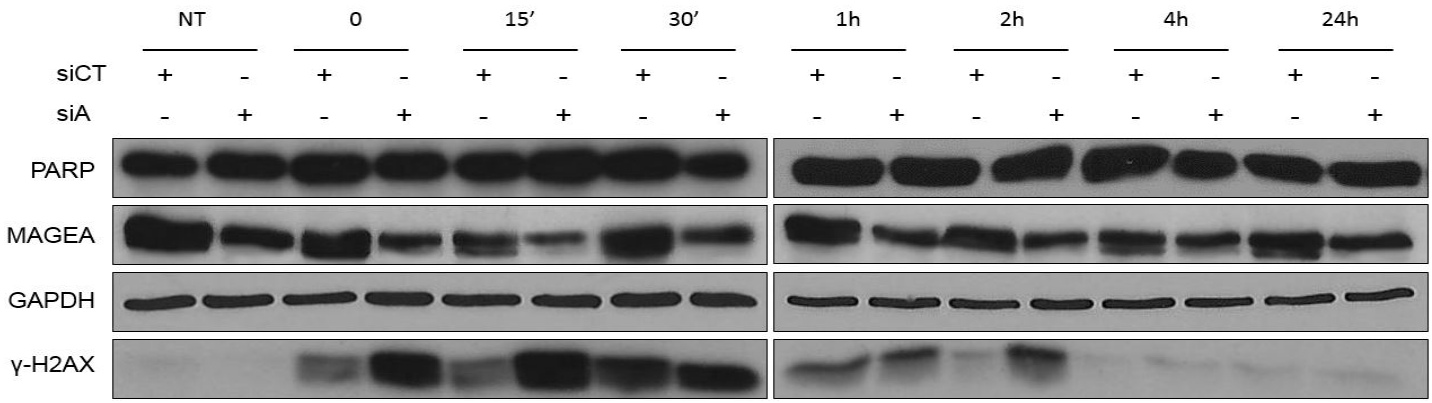

Figure 15. MAGEA knockdown affect the cell recovery after a treatment of Camptothecin in MelJuso cells. MelJuso cells were transfected with a negative control siRNA (siCT) or a MAGEA siRNA (siA) and exposed to 5 µM of Camptothecin during 4 hours or not (NT). Then the medium was replaced by a fresh one to allow the cells to recover during different times (0 min, 15 min, 30 min, 1h, 2h, 4h, and 24h). A western blot was carried out to analyze MAGEA and γ-H2AX expression. GAPDH was used as a loading control. The experiment was performed three times.

Here too we wanted to see if the impact of MAGEA depletion on cell recovery was or was not dependent on p53. The same experiment was performed three times in p53-KO MelJuso cells.

We observed similar results as above, the only difference being that at 4h the signal for γ-H2AX was always present and that it disappeared at 24h. Thus, we observed no effect of p53 KO on the cell recovery after release in MAGEA-depleted cells (Figure 16).

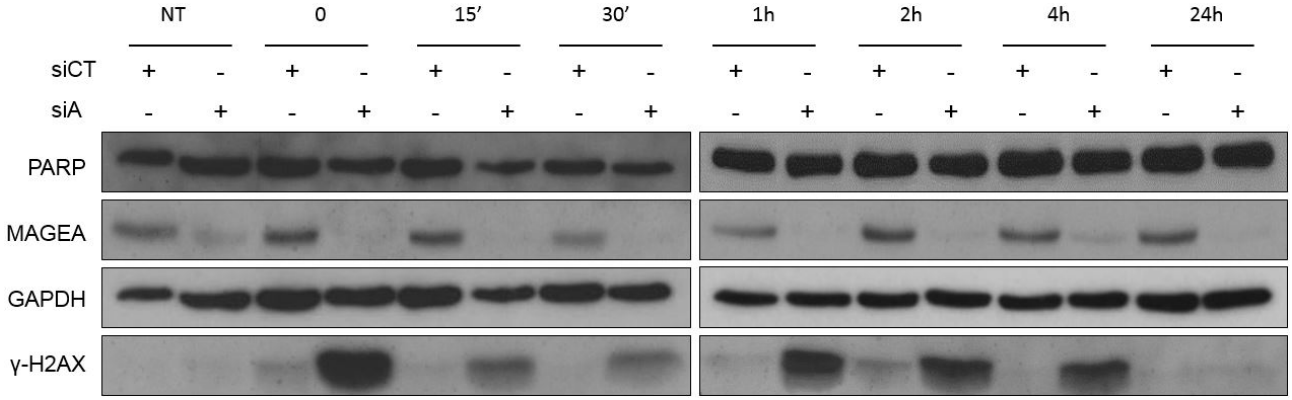

Figure 16. The role of MAGEA in genotoxic stress resistance to camptothecin is p53-independent. p53-ko MelJuso cells were transfected with a negative control siRNA (siCT) or a MAGEA siRNA (siA) and exposed to 5 µM of Camptothecin during 4 hours or not (NT). Then the medium was replaced by a fresh one to allow the cells to recover during different times (0 min, 15 min, 30 min, 1h, 2h, 4h, and 24h). A western blot was carried out to analyze MAGEA and γ-H2AX expression. GAPDH was used as a loading control. The experiment was performed three times.

2. Assessment of the role of MAGED2 in genotoxic stress resistance and DNA damage response in context of CPT treatment

Given that MAGED2 relocalizes to the nucleus following a CPT treatment (Pirlot, C. *et al.* 2016), we were also interested to determine if it had an impact on cell survival and DNA repair following genotoxic stress.

A viability assay was performed on cells treated with a Negative Control siRNA (siCT) or a siRNA against MAGED2 (siMAGE-D2). Western blot was performed in order to confirm MAGED2 depletion at 48 and 72 hour post-transfection (Figure 17A). The siRNA was very effective as nearly no MAGED2 was observed in the depleted cells.

As before, the viability assay highlighted that CPT treatment leads to a decrease of cell viability in both control cells and MAGED2 depleted cells. However, in the absence of MAGE-D2, MelJuso cells are more sensitive to camptothecin treatment. From 2,5µM to the highest concentrations tested the difference in viability between control cells and MAGED2 depleted cells is about 15%, the viability of MAGED2 depleted cells being about 50%. This difference is significative, as attested by ANNOVA (p-value < 0,0001). More precisely, there is a significant difference between the averages at 5, 10, 25 and 50 µM (Figure 17B). This experiment was performed three times. The statistical significance was determined by a two-way ANOVA and each average of each concentration was compared two by two due to Bonferroni post-test.

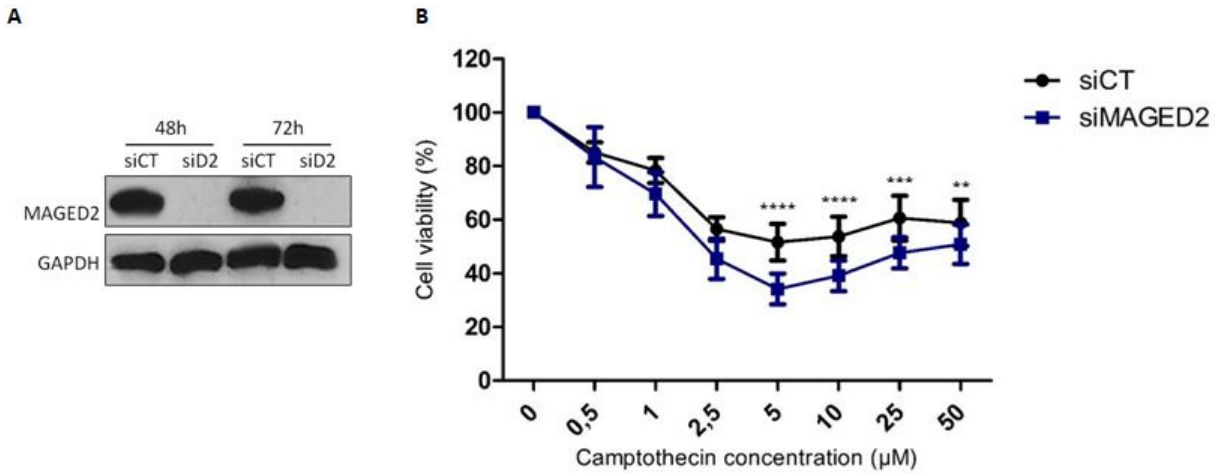

Figure 17. siRNA knockdown of MAGED2 reduces the resistance of MelJuso cells to camptothecin treatment.
(A) Western blot showing MAGED2 expression after 48h and 72h of transfection with a negative control siRNA (siCT) or a MAGED2 siRNA (siD2) in MelJuso cells. GAPDH was used as a loading control.
(B) MelJuso cells transfected with a siCT or siMAGED2 were treated with Camptothecin at the indicated concentrations. The cell viability was determined after 24 hours of treatment by a MTS assay. Each point represents the mean of three independent experiments and the error bars represent the standard error. The data were analyzed with a two-way ANOVA and a Bonferroni post-test.

We performed the same viability assay with p53-ko MelJuso cells in order to analyze whether the effect of MAGED2 in CPT resistance is dependent on p53. The knockdown of MAGED2 by siMAGED2 was confirmed by immunoblotting for the 48 and 72 hour post-transfection times (Figure 18A). This experiment was performed three times, the results being presented as a

mean of three. A two-way ANOVA allowed to determine the statistical significance and Bonferroni post-test allowed to compare each average of each concentration two by two.

The general observations are that cell viability decreases following CPT treatment both in control cells and MAGED2-depleted cells and that the profile of the two curves is quite different from those observed above in MelJuso. Indeed, whereas cell viability of control cells drops to 60% as previously, there is no further loss in viability in MAGED2-depleted cells. There is even a significant difference between the means at 0.5, 1 and 2.5 µM, the cell viability of the depleted cells being higher than that of the control cells at these points (Figure 18B).

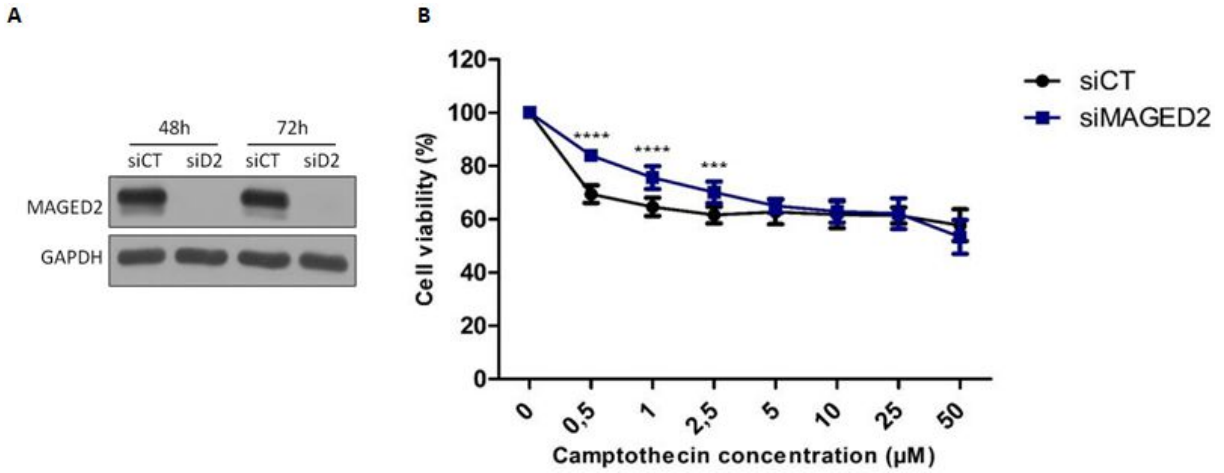

Figure 18. The role of MAGED2 in genotoxic stress resistance to camptothecin seems to be p53-dependent.
(A) Western blot showing MAGED2 expression after 48h and 72h of transfection with a negative control siRNA (siCT) or a MAGED2 siRNA (siD2) in p53-ko MelJuso cells. GAPDH was used as a loading control.
(B) p53-ko MelJuso cells transfected with a siCT or siMAGED2 were treated with Camptothecin at the indicated concentrations. The cell viability was determined after 24 hours of treatment by a MTS assay. Each point represents the mean of three independent experiments and the error bars represent the standard error. The data were analyzed with a two-way ANOVA and a Bonferroni post-test.

3. Verification of p53 KO in the MelJuso clones obtained by CRISPR/Cas9

Three p53-ko clones (E1, E12 and F2) were obtained in our lab using the CRISPR/Cas9 system on MelJuso cells. The initial idea was to delete the entire TP53 gene by targeting the beginning (exon 2) and the end (exon 9). Those three clones were the ones that were PCR positive for the large deletion. We performed a western blot to analyze the signal for p53 in those three clones and we choose E1 clones based on this. Since we used E1, it seemed interesting to determine the type of deletion induced by CRISPR/Cas9 in the TP53 gene. We started by extracting DNA from our three clones. We then performed a PCR using three specific oligonucleotides sequences, 2 of which detect the large deletion and 2 amplify the target area upstream of the gene (exon 2). The resulting PCR products were cloned in a plasmid by TA cloning. After these cloning, we sequenced with oligonucleotides on either side of the cloning site. We sequenced two clones of TA cloning for each PCR product to avoid observing a mutation that would be a PCR artifact. We analyzed the results of the sequencing with Blast, Sequence Scanner and SerialCloner.

In the wild-type, we observed a small deletion on the two allele that is in intron 3-4 but it is a polymorphism. For one allele of the E1 clone, we found a large deletion being about 100 base

pairs that extends between exon 2 and exon 9 (Figure 19a). The other allele is not mutated, it only contains a small region mutated in intron 3-4 (Figure 19b). For the F2 clone, we also observed a large deletion about 100 base pairs that extends between exon 2 and exon 9 of one of the two alleles (Figure20a). The other allele is not mutated, it only contains a small region mutated in intron 3-4 (Figure 20b). However, for the E12 clone, we did not see a large deletion but the same deletion observed in the wild-type, so this clone is not mutated (Figure 21).

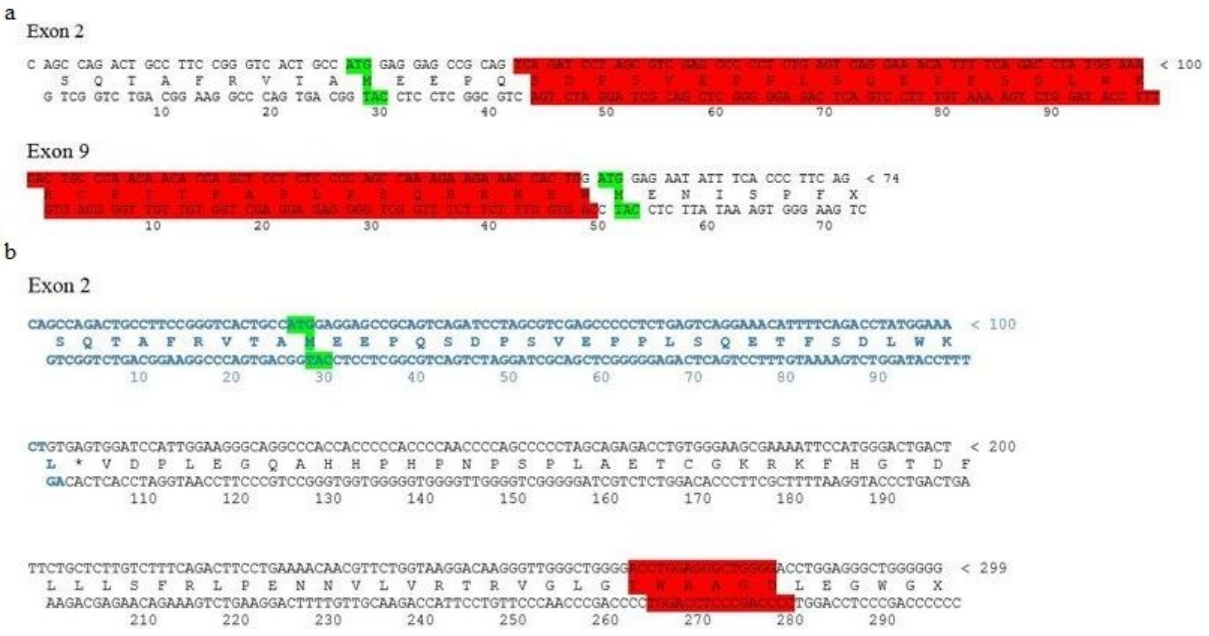

Figure 19. (a) Sequence of exon 2 and 9 of one allele and deletion (in red) from exon 2 to 9 in clone E1. (b) Deletion in intron 3-4, near exon 2 (in blue), in the second allele. The translation initiation codon is shown in green.

Figure 20. (a) Sequence of exon 2 and 9 of one allele and deletion (in red) from exon 2 to 9 in clone F2. (b) Deletion in intron 3-4, near exon 2 (in blue) in the second allele. The translation initiation codon is shown in green.

Figure 21. (a-b) Sequence of exon 2 (in blue) and deletion in intron 3-4 of one allele of clone E12; there is no deletion on exon 2. The translation initiation codon is shown in green.

4. Analysis of *MAGE* gene expression

We received from Mr. De Smet Charles an analysis of the expression of *MAGEA* genes in different cell types. In MelJuso, the most commonly expressed *MAGEAs* are *MAGEA3* and *MAGEA6*. MAGEA1, A2, A4, A10 and A12 are poorly expressed in these cells (Figure 22).

Gene ID	Gene Name	Hs 940.T	Hs 944.T	IPC-298	K029AX	MEL-JUSO	SK-MEL-28	SK-MEL-30	SK-MEL-5
ENSG00000124260	MAGEA10		72	0,3	29	0,2	6		0,7
ENSG00000147381	MAGEA4	0,3	159	0,1	11	0,1	39	0,1	0,3
ENSG00000156009	MAGEA8	0,1	3	0,1	0,1			0,1	
ENSG00000185247	MAGEA11		16	0,2	1		0,1		0,3
ENSG00000197172	**MAGEA6**	6	327	72	173	**181**	111	16	190
ENSG00000198681	MAGEA1		225	0,8	122	0,1	5	0,1	6
ENSG00000213401	MAGEA12	2	255	84	168	2	87	23	166
ENSG00000221867	**MAGEA3**	21	313	78	270	**49**	187	9	303
ENSG00000242520	MAGEA5		0,1		0,1				0,1
ENSG00000267978	MAGEA9B		0,1		0,2				0,1
ENSG00000268606	MAGEA2	0,1	2	0,6	1	0,9	0,6	0,3	2

Figure 22. Expression of MAGEA genes in MelJuso (grey column) cells and other cell types. MAGEA3 and MAGEA6 have the highest states of expression in MelJuso cells (shown in blue). The state of expression is given in TPM (transcripts per million).

V. DISCUSSION

Several studies have shown that some MAGE proteins are involved in DNA damage repair processes. Expression of some MAGE proteins promotes cell survival. Some MAGE proteins interact with DNA damage sensor proteins or with regulators involved in DDSB repair pathways (NHEJ, HR). The aim of our studies was to determine if MAGEA and MAGED2 are involved in genotoxic stress resistance and DDR. We analyzed the impact of siRNA-mediated depletion of MAGEA and MAGED2 proteins on cell survival and the DDR using CPT as DNA damaging agent on MelJuso cells.

We observed an effect on cell survival of cells depleted for MAGEA after exposure to CPT. These had a lower viability than control cells, showing that MAGEA depleted cells are more sensitive to CPT. We wondered if this stress resistance conferred by MAGEA could be related to the tumor suppressor p53. Indeed, previous studies had shown that some MAGEA protein are able to downregulate p53 activity. MAGEA2 can interact with the DNA binding domain of p53 and therefore prevent its interaction with the chromatin (Marcar, L. et al. 2010). MAGEA1 and A2 recruit histone deacetylase 3 (HDAC3) leading to hypoacetylation of the histones surrounding the DNA binding sites of p53, thus blocking p53 access and activity (Monte, M. et al. 2006). MAGE-C2 enhances TRIM28-dependent ubiquitination and degradation of p53 (Doyle, J.M. et al. 2010). We worked MelJuso cells in which the *TP53* gene had been targeted using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system. We observed no impact of p53 KO on the decreased survival, thus concluding that the resistance conferred by MAGEA is not dependent on p53.

To go further and look more directly at DNA damage and repair, we looked at phosphorylation of histone H2AX which is an early marker of DDSB. We saw a much stronger γ-H2AX response after CPT treatment in cells depleted for MAGEA. It seemed to disappear more slowly in MAGEA-depleted cells. However, it is not clear if this is due to impaired reparation or if it is just because at the beginning there is more γ-H2AX in these cells. Again, we saw no effect of p53 KO on the increased γ-H2AX response, which indicates that it is independent of p53. We wondered where MAGEA proteins could play a role and what other experiments would be interesting to try.

An explanation of how MAGEA proteins make cells more resistant to CPT could be that they protect cells from damage or are involved in DDR. Its involvement could be varied because they could allow the recruitment of repair proteins, could directly interact with proteins from repair pathways or could even act as a transcription factor regulator whose target genes are genes involved in damage repair pathways. Cells being depleted for MAGEA would no longer be able to repair the damage induced by the CPT and would therefore die faster than the control cells, which would explain the greater decrease in viability. This would also explain why there is a strongly γ-H2AX response in cells depleted in MAGEA which cannot repair their damage and therefore accumulate γ-H2AX to them.

Another explanation could be that MAGEA would modulate ATM activity. Several studies have shown that ATM is the main kinase that phosphorylates H2AX. We saw a stronger signal of y-H2AX when MAGEA is depleted so MAGEA could downregulate ATM activity which could explain that y-H2AX is much more phosphorylated when MAGEA is depleted. However, this does not explain the increased sensitivity to CPT of cells depleted of MAGEA.

MAGEA could regulate the ubiquitin-proteasome pathway for the repair of Topoisomerase I-DNA covalent complexes caused by CPT. It has been described that, after the arrest of transcription induces by Top1 cleavage complex, an ubiquitin-proteasome pathway is activated. Polyubiquitination of Top1 leads to its degradation by the 26S proteasome. The DDSB are then repair through a PARP1-dependent process (Lin, C.P. et al. 2008). MAGEA could therefore interact with the E3 ubiquitin ligase participating in ubiquitination and stimulate the ubiquitination of Top1, contributing to DDSB repair. There would be less damage and therefore less y-H2AX foci, which could easily be verified by immunofluorescence.

Other experiments would be interesting to try. We could use the analysis of *MAGEA* gene expression in MelJuso cells to focus on the *MAGEAs* most expressed there. We could then performed a rescue experiment i. e. knockdown all the *MAGEA* and express only one and do the same experiments to see if we have the same phenotypes. The main actors in cell resistance to CPT could then be defined. We could also set up a TUNNEL assay and the comet test which allow to measure DDSB in eukaryotic cells. These could show whether what we have observed (stronger γ-H2AX response in cells depleted for MAGEA) corresponds to a large number of lesions that accumulate in the cells or whether the cell has a large lesion that it not able to repair. We wanted to know in which repair system MAGEA is part of. DDSB being repaired by NHEJ and HR, we have developed an assay allowing to measure to role of MAGEA in NHEJ and HR. The recent results are not significant.

We performed the same experiments on MAGED2. We observed that cells depleted for MAGED2 are more sensitive to CPT and therefore die more. Here, the same experiment repeated on the p53-KO cells suggested that the impact of MAGED2 on cell survival is p53-dependent.

We wondered if this decrease in viability was due to a damage repair problem or if cells without MAGED2 are more susceptible to apoptosis. In other words, if MAGED2 is involved in DDR and therefore cells without MAGED2 accumulate damage and undertake apoptosis or if they are more likely to do apoptosis. The second hypothesis is supported by previous studies that showed that MAGED2 suppresses the TRAIL2 receptor and thus inhibits TRAIL-induced apoptosis in melanoma cells (Tseng, H.Y. *et al.* 2012). When MAGED2 is knockdown, the activity of p53 is increased and promotes transcriptional upregulation of TRAIL2, leading to apoptosis. This hypothesis is also supported by another study that showed that MAGED2 interacts with p53 and prevents its transcriptional function. So, an explanation could be that when cells are depleted for MAGED2, they are more sensitive to CPT because there is a repair problem or because MAGED2 no longer suppresses apoptosis by removing TRAIL2 and/or downregulating p53. But when p53 is also KO in these cells, there is a rebalance and the cells do less apoptosis despite the damage. Further research is necessary to measure apoptosis in our control and depleted cells for MAGED2 treated and untreated with CPT. We could performed an Annexin V assay which consist of Annexin V stain and an analysis by flow cytometry. We could also analyze by flow cytometry the active form of caspases or made a western blot analysis of cleaved PARP.

We also know that in the U2OS human osteosarcoma cell line, MAGE-D2 delocalizes in the nucleoplasm after a treatment with camptothecin or other drugs (Pirlot, C. et al. 2016). Since MAGE-D2 is delocalized in the nucleoplasm after a genotoxic stress, it could play a role in DNA damage repair. Experiment performed in our lab also show this relocalisation in MelJuso cells and suggest that MAGED2 attach to chromatin. The repair assay for both NHEJ and HR is

also perfumed and showed that MAGED2 depletion leads to a decrease in both NHEJ and HR. Having these results there is still a lot of research to be done on this subject.

Concerning our MelJuso cells where we targeted the *TP53* gene with CRISPR/Cas9, we need to go further because we only saw a mutation in exon 9, not exon 2. We can therefore say that it is probably this mutation that destabilizes the transcript. However, we cannot say that there is no protein at all. We could eventually do CRISPR again to make sure that the protein is no longer functional.

VI. CONCLUSION

Our aim was to further investigate the role of MAGEA and MAGED2 proteins in genotoxic stress resistance and DDR.

MelJuso cells have been shown to be more sensitive to CPT when MAGEA or MAGED2 were depleted. This therefore highlighted a role of these proteins in cellular resistance. We also showed, thanks to the DDSB marker, that MAGEA could be involved in the repair of the damage and that its role is not dependent on p53.

Despite these observations, we were unable to confirm or not the role of MAGEA and MAGED2 proteins in DDR. Other interesting research can be done to further our knowledge of these proteins and their role but we have shown a first glimpse of it.

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