

The Biological Function of Cancer-testis Antigen MAGE-C2/CT10 in Multiple Myeloma

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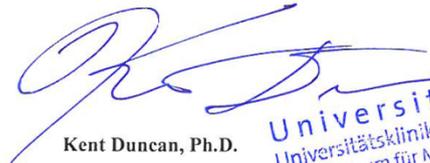
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I hereby certify as a native speaker and molecular biologist that the English language used in this thesis, while not at native speaker level, is of sufficient quality to understand the presented thesis research.


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Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

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“No amount of experimentation can ever prove me right; a single experiment can prove me wrong. “ (Albert Einstein)

To the memory of my father

ABSTRACT

Cancer-testis antigens belonging to the MAGE class I family of genes, such as MAGE-C2/CT10, are commonly expressed in Multiple Myeloma (MM). Expression of MAGE class I genes in MM is associated with aggressive disease, resistance to chemotherapy and a poor clinical outcome, suggesting that MAGE genes may confer a survival advantage on myeloma cells. MAGE-C2/CT10 is thought to be an excellent candidate for cancer immunotherapy based on its myeloma-specific expression. It was shown that MAGE-C2/CT10 enhances p53 polyubiquitination and proteasome-dependent degradation which may impair p53-dependent apoptosis. However, in spite of its frequent expression, the biological function of MAGE-C2/CT10 in malignant plasma cells has never been elucidated.

Therefore, by using the RNA interference approach, we aimed to investigate, *in vitro*, the impacts of MAGE-C2/CT10 silencing on the survival of tumor cells derived from MM patients.

We found that MAGE-C2/CT10 silencing inhibited the proliferation and anchorage-independent growth of myeloma cell lines, irrespective of the functional status of p53. The anti-proliferative effect of MAGE-C2/CT10 silencing was due to a decrease of cells in the S phase, a cell cycle arrest at both G0/G1 and G2/M transitions, and an increase in the subG0/G1 diploid population based on an initiation of apoptotic cell death. Importantly, MAGE-C2/CT10 overexpression was able to rescue the anti-proliferative effect of MAGE-C2/CT10 depletion and protected cells from apoptotic cell death. At the molecular level, the loss of MAGE-C2/CT10 expression increased the phosphorylation of the histone variant H2A.X at Ser139, which is a sensitive indicator of DNA damage, enhanced the constitutive activation of the ATM/ATR-CHK1/CHK2 DNA damage response and led to an accumulation of the endogenous level of p53 protein. The stabilization and activation of p53, through phosphorylation at Ser20 correlated with an up-regulation of p21^(WAF1/CIP1) and GADD45A α , which are the main mediators of p53-dependent and independent G1/S and G2/M cycle arrest, respectively, and a two-fold increase in the expression of the pro-apoptotic Bcl-2 genes BAX and BAK, being the effectors of p53-dependent and independent intrinsic apoptotic pathway in response to DNA damage.

Collectively, our findings, strongly, support an anti-apoptotic function of MAGE-C2/CT10 in MM through the regulation of key molecules involved in the regulation of DNA damage repair and p53-dependent and/or independent apoptotic pathways. The central role of MAGE-C2/CT10 in the biology of myeloma suggests that this CTA represents a promising target for myeloma-specific immunotherapies or other targeted modes of therapy for MM.

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ABBREVIATIONS

5-AZA-CdR	5-aza-2'-deoxycytidine
7AAD	7-Aminoactinomycin D
ASCT	Autologous stem cell transplantation
ATM	Ataxia telangiectasia mutant
ATR	Rad3-related protein kinase
BCL 2	B cell CLL/lymphoma 2
BMSC	Bone marrow stromal cells
BrdU	5-bromo-2'deoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHK	Checkpoint kinase
CSR	Class switch recombination
CTA	Cancer-testis antigens
CTL	Cytotoxic T lymphocytes
DSB	Double strand breaks
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein

GVHD	Graft versus host disease
GvM	Graft versus Myeloma
HDAC	Histone deacetylases
HLA	Human leukocyte antigen
HMCL	Human Myeloma Cell Lines
HRD	Hyperdiploid
Id	Idiotypic
IFN	Interferon
IgH	Immunoglobulin Heavy chain
IgL	Immunoglobulin Light chain
IL	Interleukin
M protein	Monoclonal immunoglobulin protein
MAGE	Melanoma Associated Antigen-A1
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Murine double minute 2
MGUS	Monoclonal gammopathy of undetermined significance
MHD	MAGE homology domain
miRNA	MicroRNA
MM	Multiple Myeloma
mRNA	Messenger RNA
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

NF-κB	Nuclear factor-kappa B
NHRP	Non-hyperdiploid
NY-ESO-1	New York oesophageal squamous cell carcinoma 1
P2A	porcine Teschovirus_1 virus 2A ribosomal skipping peptide
PARP	Poly ADP-ribose polymerase
PBMC	Peripheral blood mononuclear cells
RFP	Red fluorescent protein
RNAi	RNA interference
SCP1	Synaptonemal complex protein 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEREX	Serologic analysis of recombinant cDNA expression libraries
SHM	Somatic hypermutation
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMM	Smoldering Multiple Myeloma
SSX	Synovial sarcoma X
TBST	Tris-buffered saline with Tween 20
VCAM1	Vascular-cell adhesion molecule 1

CHAPTER 1: INTRODUCTION

I. Multiple Myeloma

Multiple myeloma (MM) is a malignancy of terminally differentiated B-lymphocytes that accounts for 1 to 2% of neoplastic diseases and is the second most common haematological malignancy after Non-Hodgkin lymphoma [1]. MM is characterized by a very slowly proliferating monotypic plasma cell population, which localizes to the bone marrow, produces excessive amount of monoclonal Immunoglobulin, called the M protein (IgG or IgA), found in the serum and the urine of MM patients and used as a marker for the diagnosis [2, 3]. The accumulation of malignant plasma cells in the bone marrow can lead to lytic bone lesions, impaired haematopoiesis and the deposition of M protein in the kidney. Consequently, MM patient will suffer from a variety of myeloma-related complications, such as bone fractures, hypercalcemia, anemia, renal failure, and increased susceptibility to infections [4]. Myeloma is still considered incurable and almost all patients will die from the malignancy within a time frame of only 4-5 years [5].

I.1 Epidemiology and risk factors

Although, in most cases, individuals with MM have no apparent risk factors, MM is not caused by a single factor, but certain factors acting together may increase the risk of developing it. Epidemiological studies have shown that the risk of MM increases with age and is more common among male particularly African American [5]. An involvement of the lifestyle factor has been proposed, because of higher risk of MM in obese people and an inverse association with high vegetable and fish intake [6]. Case control and cohort studies demonstrated that lifetime exposure to chemical used in agriculture and in petroleum industries as well as high doses radiation are possible risk factors [7-9]. The observation that sheep farm residents are at risk may indicate the involvement of certain zoonotic viruses in the development of MM [10]. Although a history of chronic antigenic stimulation and autoimmune disease has not been consistently related to MM, elevated myeloma incidence was observed in patient with rheumatoid arthritis [11]. According to a number of studies, there is a link between chronic viral infection with hepatitis C virus and the development of MM [12]. The risk of developing this type of cancer also appears to be higher among people living with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS)[13]. Although the risk of developing other types of cancer is higher among people with HIV/AIDS, it is not clear whether these associations are due to a weakened immune system or to HIV

related cancers such as Kaposi-sarcoma-associated herpes-virus, found in cultured non-malignant bone marrow Dendritic cells from MM patients and Bone Marrow biopsies [14, 15]

A number of studies have reported an increased risk of developing MM for subjects with family history of MM [16] and lymphohematopoietic cancer [17] suggesting an important role of the genetic component in the etiology of MM. Risk of MM has been associated with three polymorphic microsatellites in the promoter region of IL10 which is involved in the inflammatory response and may promote myeloma cell survival. This genetic variation appeared to increase the expression level of IL10 [18]. Significant association was reported between the genetic variants of the Non-homologous end joining DNA ligase IV and the predisposition to MM which is characterized by aberrant immunoglobulin class switch recombination [19].

Frequently, the onset of MM is preceded by an asymptomatic phase during which the blood level of the M protein is unusually high but not enough to diagnose the malignancy. This asymptomatic plasma cell proliferative disorder is called "monoclonal gammopathy of undetermined significance" (MGUS) and can last a lifetime in a discrete state of expansion of monoclonal transformed plasma cell population and does not exceed 10% of the marrow cells. It is a hundred times more common than multiple myeloma, occurs in ~1-3% of person aged of 50 and increases to ~3-5% of people over 70 years [20]. Because subjects with MGUS have an increased risk of progression to multiple myeloma (1% per year), they are subject to regular medical monitoring to detect any changes [21]. In some patients, an intermediate asymptomatic but more advanced premalignant stage, referred to smoldering multiple myeloma (SMM), is clinically recognized with the full-bore MM [22]. However, the molecular basis for the progression from MGUS to MM remains largely unknown.

I.2 The pathophysiology of MM

I.2.1 B cell ontogeny

B cells develop from a pool of multi-potent stem cells in the bone marrow and the different steps of differentiation are characterized by the differential expression of marker proteins and the stepwise recombination of the immunoglobulin gene loci [23]. It is the locus of the Immunoglobulin (Ig) heavy (H) chain that rearranges the first, at pro-B-cell stage and this rearrangement proceeds in two stages. The assembly of D_HJ_H operates first, followed by V_H segment to D_HJ_H . In-frame V(D)J recombination gives rise to $Ig\mu$ chain, which is expressed in

the cytoplasm and on the surface of pre-B cells associated to an invariant substitute of the Immunoglobulin light chain (IgL), VpreB- λ_5 . The expression of μ -VpreB- λ_5 complex by pre-B cells appears to be essential as it delivers a positive signal for the survival, the proliferation and the initiation of IgL recombination. This latter operates on a single locus κ or λ -chain of IgL. It starts on a first locus of the chain κ , where the first segment V κ joins J κ segment. When an IgL locus is rearranged in frame, the pre-B cell can therefore assemble the functional IgH-IgL and express on the surface a complete IgM called the B-cell receptor (BCR) and at this stage the B cell is still immature [24]. These rearrangement events are mediated by the recombinases RAG-1 and RAG-2 which are active in early and late pro-B cells as well as pre-B cells and generate double strand breaks between gene segments at specific recombination signal sequences (RSS) [25]. The DSBs are repaired by recruitment of DNA repair enzymes that mediate Non-homologous end-joining (NHEJ), TdT (terminal deoxy-nucleotidyl transferase), involved in the random addition of non-templated nucleotides called “N-nucleotides” and DNA ligase IV [26]. Immature B cells or Naïve B cells, having successfully completed Ig recombination, leave the bone marrow and migrate to the secondary lymphoid organs, where they localize in structures called follicles. Once activated by a cognate antigen presented by helper T cells, follicular B cells migrate to the germinal center where they undergo affinity maturation of their antibody through somatic hypermutation (SHM) and apoptosis for the selection of B cells producing highly specific and avid antibodies [27]. The functionality of these antibodies is further increased in the germinal center, by class switch recombination (CSR) which produces antibodies of different immunoglobulin isotypes with different functional characteristics. The germinal center reaction gives rise to non-secreting memory B cells and antibody secreting plasmablasts. These latter leave the germinal center, develop into terminally differentiated plasma cells secreting high affinity antibody and home to the bone marrow niche where they receive survival signals from stromal cells via cell adhesion molecules and provide serological memory for many years [28].

I.2.2 Origin of MM cells

Because myeloma cells have a plasmablast morphology, it is thought that they may originate from post-germinal B cell clone, which can be a memory B cell or plasmablast clone that underwent a SHM and CSR given the IgG or IgA isotype of the paraprotein and the infiltration of the bone marrow [29]. However, controversy about the MM origin still persists because some studies have evidenced in certain cases the presence of myeloma related

idiotypic determinants in B cells expressing low affinity cell surface IgM and IgD [30] and in cytoplasmic μ pre-switch B cells, found at a low amount in the peripheral blood of MM patients [31]. These findings may imply that the clonal oncogenic events occur at earlier stage of B-cell differentiation than plasma cell stage, likely pre-B cell stage and may not be the consequence of antigenic stimulation [30]. Others, however, brought evidences showing that MM clones may originate from pre-switched but somatically mutated B cells [32]. The transformation of pre-B stem cell precursor or even a multipotent hematopoietic progenitor not committed to any lineage has been also proposed based on the observation that myeloma patients bear T cell clones expressing antigenic receptors sharing the same idiotypic determinants of the immunoglobulin produced by the B cell clones [33] and express on the surface of myeloma cells the same clonal markers of myeloid, erythroid and megakaryocytic lineage [34].

Probably one of the most important finding regarding the clonal origin of MM clone is the absence of $V_H.4-21$ gene in the functional repertoire of Immunoglobulin heavy chain variable region of MM cells. $V_H.4-21$ gene is a member of the V_H4 family, encodes for an auto-antibody and is over-presented in B cell repertoire before the clonal deletion associated to antigen selection in the germinal center [35]. In pathological states, $V_H.4-21$ gene rearrangements are increased like in auto-immune diseases (cold agglutinin disease and systemic lupus erythematosus) as well as in some B cell neoplasms (chronic lymphocytic leukemia, diffuse large lymphoma, and acute lymphocytic leukemia). However, unlike these B cell neoplasms, that arrest at earlier stages of B cell ontogeny, MM clones lack $V_H.4-21$ gene rearrangement which may, at first, exclude any auto-immune etiology of the disease but also indicate that MM clones have been selected by antigen exposure and may originate from a memory B cell precursor [36]. This hypothesis is consistent with the highest rate of somatic mutation (a process that is activated only when B cells enter the memory compartment and is limited in time) and with the absence of intraclonal variation within the Immunoglobulin heavy chain variable region of MM cells which defines the clonal character of this disease [29]. The malignant transformation of a memory B cell clone might be closely related to an accidental chromosomal rearrangement (translocation), which may promote an abnormal proliferation by connecting an oncogene to enhancer sequences specially activated in B cell lymphoid lineage such as immunoglobulin gene. This chromosomal accident is likely determinant in the immortalization of the memory B cell clones. Although the pathogenesis of MM is still poorly understood, both in terms of the role of stimulus as well as the nature of

oncogenes being involved in the initiation of this immuno-proliferative disease, it was suggested that myeloma progeny acquires early key genetic events at the germinal center, due to errors associated with the repair of DNA double-strand breaks (DSBs) that are naturally introduced in the IgH loci during the physiological processes necessary to generate antibody diversity namely SHM and CSR [37]. It should be noted though, that in contrast to the homogeneity of MM plasma cell clone, the premalignant MGUS disorder shows evidence of intraclonal heterogeneity suggesting the persistence of mutational activity in the germinal center which induces slightly mutations in the same unique clonal IgH rearrangement as the progeny matures [38]. The acquisition of additional genetic hits over time mediated through the loss of heterozygosity, gene amplification, mutations, epigenetic and microRNA changes by the one of the sister clones initially generated during the germinal center reaction may lead to the progression to the clinically recognized features of MM. This clonal evolution is thought to occur in a non-linear fashion. Genetic abnormalities are acquired randomly and the emergent clone is selected based on the clonal advantage it confers and this what gives rise to the number of sub-clone ecosystems corresponding to the different phases of the disease during the progression from MGUS to MM and also to the differential responses to treatment [39].

I.3 Genetic events of MM pathogenesis

I.3.1 Chromosomal translocations

One of the most important immortalizing event involved in the development of MM are translocations involving the IgH on chromosome 14 (14q32), present in 50% of the premalignant MGUS, 60% of fully malignant MM tumors and 90% of human myeloma cell lines (HMCL) [40, 41]. Each translocation is associated to the activation of a proto-oncogene juxtaposed to an IgH chain enhancer. Unlike lymphoma, in which translocation partner is specific of the histological type; chromosomal partners are extremely diverse in MM. To date, more than 30 chromosomal regions have been reported, some of them seem to be recurrent. It has been found that 40% of MM patients show five recurrent reciprocal IgH translocations including t(11;14) (CCND1; 14%), t(4;14) (FGFR3, MMSET; 11%), t(16;14) (MAF; 3%), t(14;20) (MAFB; 1.5%), t(6;14) (CCND3; <1%)[39]. These simple reciprocal translocations occur at breakpoint site localized on 14q32, in the centromeric switch region, downstream of the VDJ region and upstream of the constant genes. It has been proposed that defects in the DNA modification process associated mostly to CSR process, less often to SHM and rarely if

ever to VDJ recombination, can cause DSBs or mutations leading to these early initiating oncogenic events and pushing cell towards transformation [37, 42]. Besides 14q32 translocation, other primary chromosomal breakpoints were detected at 16q11 and 22q11 sites [43], whereas 15 to 20% of MM patients present other unique chromosomal translocations, which may suggest a random origin of these non-recurrent rearrangements [44]. However, whether involving the IgH locus or not, these primary translocations do not seem to be sufficient for the malignant progression of the disease. Likely, other genetic alterations involving proto-oncogenes and tumor suppressor genes are needed for the emergence of the fully-bore malignant plasma cells.

Later during disease progression, other secondary IgH translocations occur in 3% of MM and are, in contrast to primary translocations, independent of the DNA modification processes which are inactive in mature myeloma progeny [40]. A typical secondary translocation is t(8;14) that lead to MYC deregulation and is often complex and non-reciprocal translocation or insertion juxtaposing MYC gene and E α enhancer sequences with the involvement of 3 chromosomes and sometimes with associated insertion, duplication [45]. Myc translocation is absent or rare in MGUS but occurs in 15% of MM patients, 40% of advanced disease, 90% of HMCL and is associated to more aggressive transformation. Nonetheless, MYC translocations do not always involve IgH gene, other partner genes poorly characterized seem to be involved suggesting that other non-IgH enhancer regulatory sequences might up-regulate MYC gene expression in MM [46].

I.3.2 Aneuploidy in MM

Other major genetic abnormalities seen in MM tumors are numeric chromosomal abnormalities. Nearly half of the tumors are non-hyperdiploid (NHRP) and are further subdivided into three groups, including hypodiploid (≤ 44 chromosomes), pseudo-diploid (45-46 chromosomes) and near tetraploid (>75) which appears to be 4N duplication of hypodiploid and pseudo-diploid karyotype cells [47]. In addition, NHRD tumors are often associated with high prevalence of the recurrent primary IgH translocations and chromosome 13 monosomy or partial deletion [48]. The remaining MM tumors (50 to 60%) are hyperdiploid (HRD) (48-74 chromosomes) with multiple trisomy of the odd numbered chromosomes (3, 5, 7, 9, 11, 15, 19 and 21) [49], which are thought to be acquired through a single mitotic catastrophe rather than a stepwise gain of chromosome over time [50]. Unlike NHRD, IgH translocations are less common in HRD tumors, which may possibly explain the

favorable overall survival of this cytogenetic group in comparison with NHPD MM tumors [47, 51]. Since 14q32 chromosomal translocations are almost incompatible with hyperdiploidy, this latter constitutes probably a distinct oncogenic pathway in MM from NHRD [51]. Nonetheless, both 14q32 chromosomal translocations and hyperdiploidy have been demonstrated in MGUS, which shows the early onset character of these anomalies [52]. It remains unclear, however, which genetic abnormality occurs first during the natural history of MM.

I.3.3 Cyclin D dysregulation

Between the two oncogenic pathways described above the dysregulation of Cyclin D group appears as an early and unifying pathogenesis event between these two pathways, present in all MM tumors including the premalignant MGUS tumors and lead to the dysregulation of G1/S transition and consequently to the emergence of these low proliferative malignant plasma cells [53]. Consistent dysregulation was first noted as a consequence of t(11;14) and t(6;14) which affect directly CCND1 and CCND3, respectively [54, 55]. Non-translocation based up-regulation of CCND also occurs in the case of t(14;16) associated to MAF up-regulation which induced CCND2 expression by binding directly to its promoter [56]. However, the mechanism of the unusual high expression of CCND2 in MM tumors with t(4;14) and with hyperdiploidy (without IgH translocation) remains unknown [53]. It is certain, though, that the absence of CCND up-regulation in some percentage of tumors is mainly due to the deletion of RB that is normally suppressed by inhibitory phosphorylation mediated by CCND members and cyclin-dependent kinase 4 and 6 to allow the progression through G1/S transition [53].

I.3.4 Chromosomal deletions and gains in MM

➤ Chromosome 13 deletion (13q14)

As in previous genetic anomalies, chromosome 13 complete or partial deletion has been described in MGUS [57] with the similar incidence described in MM (40-50%) [58]. The distribution of this genetic abnormality does not seem to be random. While, they are rarely found in hyperdiploidy tumors, they occur most frequently in NHRD MM tumors (70%) and they are constantly associated to the unique translocation t(4;14) involving MMSET and FGFR3 genes, but not the opposite. These observations suggest that chromosome 13 deletion may occur prior to the translocation event and may constitute an important factor in the initiation of the disease [58]. Interestingly, it has been shown that the Retinoblastoma protein

(RB) is the target gene for this deletion. RB is a tumor suppressor gene that suppresses tumorigenesis by regulating the G1/S cell cycle transition, whose the deregulation constitutes one of the early molecular abnormalities in MM [39]. It should be noted also, that Rb protein down-regulates IL6 gene expression which is the main growth factor in MM [59].

➤ **Gain of chromosome 1q21**

The gain of additional copies of the long arm of chromosome 1 is the fourth most frequent genetic abnormality in MM. Nonetheless, it does not appear to be myeloma specific since its occurrence has been documented in other malignancies, including hematological and solid ones [44]. Amplification of 1q21 is low in MGUS and increases to 43% during the progression from SMM to MM. This frequency is further increased to 70% in patients with translocations t(4;14) and t(16;14) and at relapse. Because the amplification of 1q21 is associated with both disease progression and poor prognosis, it is thought that this genomic region may encode for proteins that may play an important role in disease progression rather than initiation [60]. According to previous studies, CKS1B, BCL9 and PDZK1 might be crucial genes associated to this amplification [61, 62]. Indeed, fish analysis has shown, that 1 to 4 copy amplification of 1q21 is associated to the overexpression of CKS1B which promotes cell cycle progression by targeting the cyclin-dependent kinase inhibitor p27^{KIP1} for proteolysis [61].

➤ **Loss of chromosome 1p**

The deletion of the short arm of chromosome 1 is a common recurrent genetic event in MM that has been observed in 32% of MM patients [63]. 1p deletion leads to recurrent homozygote deletion of the minimal region 1p32.3 which contains two genes CDKN2C and FAF1, identified as potential targets for this deletion [64]. Deletion of 1p32.3 is associated with impaired survival in patients treated with high dose therapy and autologous stem cell transplantation. CDKN2C is an important negative regulator of G1/S transition and its loss may deregulate this checkpoint which may point to an important role of this gene in the pathogenesis of MM [64].

➤ **Loss of chromosome 17p**

The last recurrent genetic abnormality, probably biologically important in MM is the hemizygous deletion of the short arm of chromosome 17 in which maps the tumor suppressor gene TP53 (17p13), thought to be the relevant deregulated gene in this case. Fish analysis carried on in a number of studies has shown the loss of this gene in 10% of MM cases but exceptionally in MGUS cases and is associated with a shorter survival. In the absence of 17p

deletion, this gene is rarely mutated in newly diagnosed MM patients and even at later stages the mutation rate is low but rises to 25-37% in MM cases with 17p deletion [65, 66]. If we assume that the loss of one allele would require a mutation on the second allele to have a biological significance, this would argue in favor of another gene candidate other than TP53, which remains to be discovered.

I.3.5 Deregulated cell signaling pathways in MM

Whole genome and exome sequencing of a number of MM tumors detected sets of mutations as well as chromosomal rearrangements in multiple members of a given pathway that have a biological impact on the normal functioning of the pathway rather than of a specific gene [67].

➤ The activation of the nuclear factor- κ B (NF- κ B) pathway

NF- κ B is a transcription factor that plays a central role in infection, inflammation, lymphopoiesis particularly in B cell and plasma cell development [68]. In MM, NF- κ B is up-regulated in myeloma tumor cells and in the bone marrow stromal cells. While, in malignant B cells, NF- κ B confers resistance to apoptosis, in stromal cells it triggers the secretion of IL-6 and BAFF cytokines, known as growth factors that induce paracrine stimulation of MM cells [69, 70]. NF- κ B pathway is aberrantly activated in 17% of MM patients and 40% of HMCL through point mutations and homozygous deletion of BTRC, CARD11, CYLD, IKBIP, IKBKB, MAP3K1, MAP3K14, RIPK4, TLR4, TNFRSF1A, TRAF3, BIRC2, BIRC3 and CD40 [67].

➤ The activation of the mitogen-activated protein Kinases (MAPK) pathway

The MAPKs consist of three major serine threonine kinases : the p38 Map kinase family, the extracellular signal-regulated kinase (Erk) family, and the c-Jun NH2-terminal kinase (JNK) family which participate to the generation of various cellular responses, including gene transcription, induction of cell death or maintenance of cell survival, malignant transformation, and regulation of cell-cycle progression [71]. In MM, RAF/MEK/ERK pathway can be activated by the tumor microenvironment for instance the growth factor IL-6 secreted by myeloma in autocrine and paracrine manner and also by oncogenic events [72]. Because RAS is an upstream activator of RAF, RAS mutations may lead to constitutive activation of RAS and consequently to ERK pathway activation, triggering thus a proliferative signal. RAS mutations are rare in MGUS and have been noted in 30 to 50% of MM and HMCL [73]. The prevalence of these mutations increases with advanced stages of the disease. In addition, mutations in the ERK pathway involve N-RAS and K-RAS in 24 and 27% of cases as well as

BRAF in 4% of tumors suggesting that ERK pathway is important for myeloma development [39].

➤ **The activation of phosphatidylinositol 3-kinase (PI3K) pathway**

PI3K pathway regulates cell proliferation and tumor formation and its deregulation is thought to play an oncogenic role in MM because the cascade is constitutively active in MM and its inhibition induced apoptosis [74]. While, PI3K can be activated by IL-6 and IGF-1 growth factors [75, 76], it is inactivated by the phosphatase and tensin homologue tumor suppressor gene (PTEN). PTEN regulates negatively PI3K mediated phosphorylation of AKT and BAD which suppresses apoptosis. Inactivating mutations and deletion of PTEN have been reported, however because of their low prevalence in primary MM tumors, the pathogenic role of these alterations remain unknown [77].

➤ **The activation of JAK/STAT pathway**

JAK/STAT pathway is one of the most critical intracellular signaling pathways that transduces extra-cellular signal of many cytokines receptor systems to the nucleus in order to induce gene expression. The binding of a given cytokine to the receptor leads to a transient phosphorylation and activation of the receptor and JAK that serves as a docking site for STAT and adaptors linking the receptor to MAPK, PI3K/AKT and other cellular pathways [78]. The dysregulation of JAK/STAT signaling pathway has been described in MM because of aberrant IL-6 signaling which triggers a constitutive phosphorylation of STAT3 [79] and confers an anti-apoptotic signal to MM cells through an over-expression of Bcl-2, Bcl-xL and Mcl-1 [80]. JAK/STAT pathway is constitutively activated in 48% of primary myeloma tumors [63]. This activation seems to be strictly IL-6 dependent since no activating mutation in JAK or STAT has been reported in MM so far.

➤ **The Unfolded Protein Response (UPR) Pathway**

The UPR is a cascade of intracellular stress signaling events activated in response to Endoplasmic reticulum (ER) stress due to an accumulation of misfolded or unfolded protein in the lumen of the ER. In tumor cells, the high proliferation rate increases the activity of the ER, including protein folding, assembly and transport which may cause the activation of the UPR because of stress conditions associated to the unfriendly tumor microenvironment such as hypoxia, nutrient deprivation and DNA damage [81]. The UPR is activated in cancer cells to rescue stressed cells either by halting cell cycle progression and inducing ER chaperon expression to allow folding of the excess of protein in the ER. If the stress is excessive, an apoptotic cell death is induced in order to get rid of damaged cells [82]. It has been shown that

proteasome degradation and autophagy are other mechanisms activated for the clearance of protein overload in the ER [83]. The UPR relies on 3 signaling pathways, two of them rely on the transcription factors XBP1 and ATF6 which mediate the expression of the ER chaperone [84, 85] and a third signal pathway controlled by PERK/eIF2 α which blocks protein translation by phosphorylating the eukaryotic initiation factor eIF2 α [86]. In normal and malignant plasma, the abundant secretion of Immunoglobulin requires a high ability to correctly fold nascent antibodies, which may suggest an important role for UPR to the survival of MM cells. Indeed, an abundant expression of XBP1 is observed in MM and the overexpression of XBP1 in B cell transgenic mouse model under the control of immunoglobulin V_H promoter and E μ enhancer facilitates the spontaneous development of MM [87]. Moreover, the pro-apoptotic effect of Bortezomib, which suppresses 26S proteasome degradation of misfolded proteins, demonstrated *in vitro* and in clinical trials, proves the susceptibility of MM cells to the ER stress induced apoptosis [88].

I.3.6 Epigenetic deregulation in MM

➤ DNA methylation

In addition to mutations and translocations, alterations in DNA methylation are common in malignancies. Early studies showed that tumor cells are characterized by two kind of methylation defects: (i) a global hypomethylation usually involving repeated DNA sequences and transposable elements which may cause the disruption of adjacent gene expression (ii) de novo hypermethylation occurring at CpG dinucleotide islands present at higher frequency in promoter region of tumor suppressor genes which may lead to their transcriptional inactivation [89]. A DNA methylation profiling analysis of MGUS, SMM, MM and normal plasma cells identified hypomethylation events to be occurring early in the development of MM and increasing during disease progression, while DNA hypermutation events are relatively rare [90]. Consistently, others have shown by genome global analysis of MM methylation status that both hypomethylation and hypermethylation occur during plasma cell neoplasia and at the transition from MM to plasma cell leukemia stage, some hypomethylated genes are remethylated. Gene ontology analysis of the hypermethylated genes at the transition to MM showed that the epigenetic events involve many genes regulating developmental processes, cell cycle and transcription. Interestingly, the hypermethylated genes, at the transition from MM to plasma cell leukemia, are involved in the regulation of cell signaling, cell differentiation and cell adhesion molecules, which may contribute to the ability of MM cells to proliferate outside of the bone marrow microenvironment manifesting the

extramedullary myeloma and plasma cell leukemia stage. Moreover, the DNA methylation status seems to be defined by translocation and hyperdiploidy with t(4;14) having the most frequent DNA hypermethylation compared with other cytogenetic groups [91]. This could be in part related to MMSET overexpression encoding a histone methyltransferase transcriptional repressor which mediates H3 lysine 36 methylation [92]. MMSET deregulation may lead to histones modifications that promote cell survival, cell cycle progression and DNA repair [93, 94].

➤ RNA processing

MicroRNAs (miRNAs) are short non-coding RNAs of 22 nucleotides in length that control gene expression by translational inhibition and mRNA target degradation through RNA interference machinery by base pairing to partially or fully complementary sequences [95]. In addition to their role in the regulation of development, differentiation, apoptosis, stress response in normal cells [96], miRNAs might be involved in tumorigenesis because of their alterations observed in various type of cancer. It seems that miRNAs may act as oncogenes by modulating oncogenic, tumor suppressor and metastatic pathways including cMYC, p53 and RAS [97, 98]. The aberrant miRNA levels are caused by genetic, epigenetic, transcriptional and post-transcriptional modifications associated to cellular transformation [99]. In MM, miRNA expression seems to be associated with disease pathogenesis because several miRNAs have been found deregulated (up or down-regulation) in MM and MGUS in comparison to normal Plasma cells. Additionally, miRNAs such as mir-16 was found to be highly expressed in MM than in MGUS, which may suggest an association with disease progression [100]. More importantly, distinctive miRNA signatures in MGUS and MM have been identified by miRNA microarrays through the overexpression of a number of miRNAs with known oncogenic activity such as cluster mir17~92 specifically up-regulated in MM, which may suggest a potential role in the transition from MGUS to MM. In line with these findings, a genome-wide specific miRNA expression pattern was associated with different cytogenetic subtypes in comparison with normal plasma cells which may give a connection between clinical behavior and biological features of MM. miRNA clusters were differentially expressed with t(4;14), t(14;16), t(11;14) and RB deletion. Some of these deregulations were common like miR-214 and miR-375 down-regulation, others, however, represented a singular signature characteristic to cytogenetic subgroup like miR-1 and miR-133a specifically up-regulated in t(14;16) MM subgroup. Moreover, the analysis of the relationship between miRNA expression and their respective target genes showed a conserved inversed correlation

between several miRNAs deregulated in MM cells and the up-regulation and the down-regulation of genes involved in proliferation (t(14;16) and CCND2) and cell death (monosomie 13), respectively [101]. Some of other up-regulated miRNA in MGUS and/or MM seem to target survival pathway. For instance, miR-181a and b, and miR-106b~25, found in both MM and MGUS, target p300-CBP-associated factor, which is a positive regulator of p53. Being preferentially over-expressed in MM, miR-19a and b target SOCS-1, which is a negative regulator of IL-6R/STAT-3, [102]. The whole genome sequencing of myeloma showed also that the deregulation of miRNA can be effected by mutations of the RNA silencing regulators [67] that alter the DORSHA and DICER activities, the master regulators of RNA silencing machinery [103].

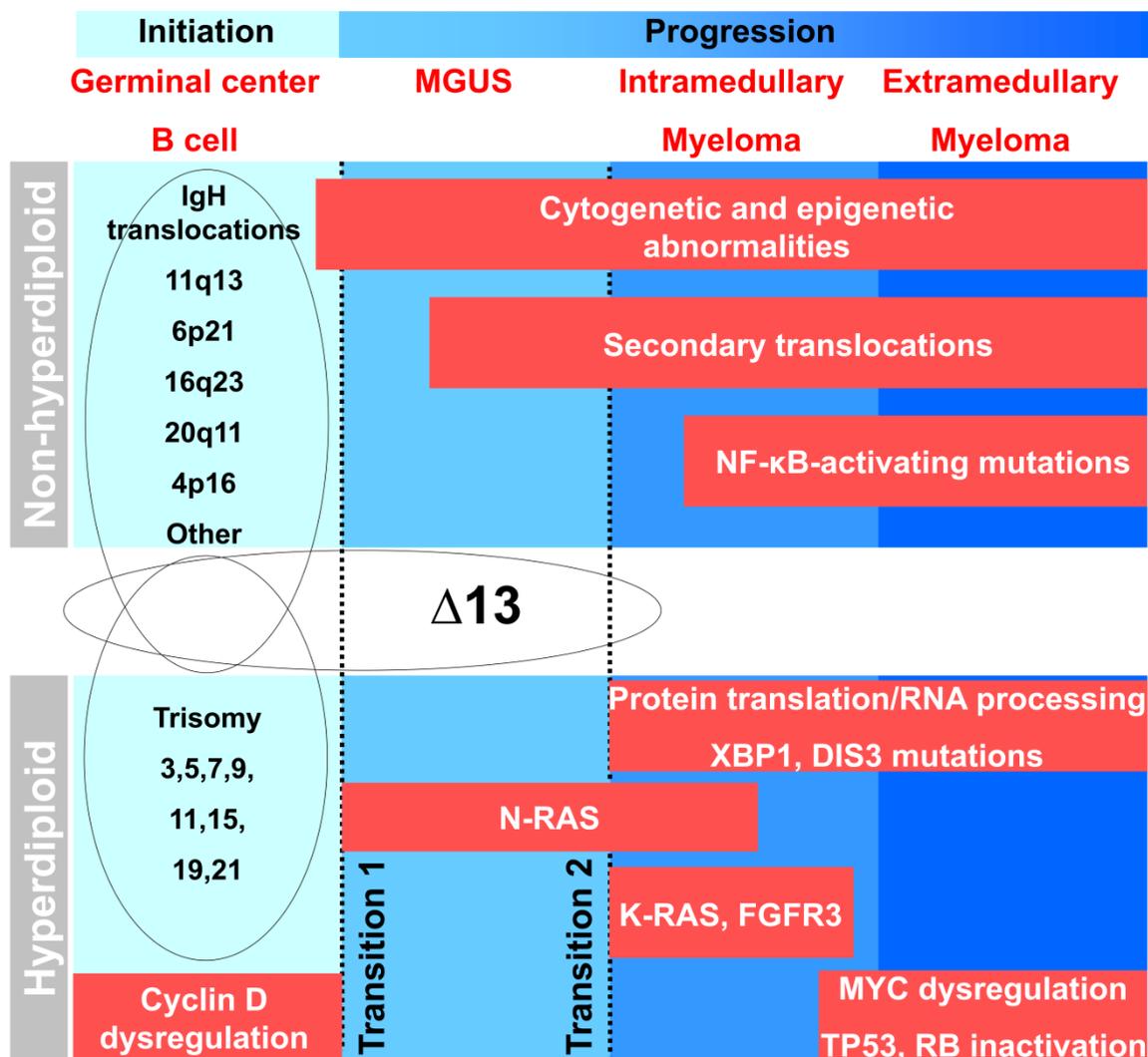


Figure 1. Model for the molecular pathogenesis of MGUS and MM. The early onset of the disease is characterized by two distinct and non-overlapping oncogenic pathways consisting of the IgH translocation/NHPD and HDP cytogenetic groups, common to MGUS and MM. Between these two pathways, the dysregulation of Cycline D group appears as an early and unifying pathogenesis event. The transition from MGUS to MM is associated with secondary translocations involving MYC. MYC

deregulation, RAS mutations and p53 mutations or deletions are associated with disease progression and drug resistance. (Adapted from Bergsagel and Kuehl, J Clin Invest, 2012)

I.4 Impact of the bone marrow microenvironment on MM progression

Although the acquisition of chromosomal translocations, hyperdiploidy, chromosomal trisomies and oncogenic off-target mutations lead to the immortalization of myeloma propagating cells, their survival is, in the first place, dependent on the bone marrow microenvironment where they migrate and continue to evolve [37, 104]. The genetic abnormalities acquired by plasma cells modify the microenvironment through adhesion-mediated autocrine and paracrine activation of various cytokines to create a niche which supports the long time survival and the accumulation of myeloma clones by the activation of growth, survival, migration and drug resistance mechanisms [105]. The adhesion of MM to bone marrow stromal cells (BMSC) via an interaction of their respective receptors, very late antigen 4 (VLA-4) and vascular-cell adhesion molecule 1 (VCAM1), respectively triggers within BMSC the activation of NF- κ B signaling pathway, the secretion of IL-6, vascular endothelial growth factor (VEGF) and receptor activator of NF- κ B (RANKL) [106, 107]. IL-6 is a key growth factor in MM secreted also by osteoblasts and MM cells perse. IL-6 promotes growth by suppressing the anti-proliferative activity of the cyclin-dependent kinase inhibitors p21 (CDKN1A) and p27(CDKN1B) through PI3/AKT pathway and up-regulates the anti-apoptotic proteins Mcl-1 and Bcl-X_L through the activation of JAK/STAT3 pathway [75, 108]. While, VEGF may promote MM growth and expansion by promoting angiogenesis [109], RANKL binds to its receptor expressed by osteoclasts, suppresses osteoclast apoptosis and stimulates their differentiation and activity which leads to bone resorption [110]. On the other hand, osteoblast activity can be suppressed by plasma cell production of dickkopf homolog 1 (DKK1) which may decrease the differentiation from precursors to mature osteoblasts by antagonizing the wingless (WNT) pathway [111]. Accordingly, the imbalance between bone resorption and new bone formation, which controls the amount of bone tissue, is lost and may result in bone destruction. Unlike other cancers, the development of osteolytic lesions is unique to MM and typical of fully malignant MM stage, a process that is associated to the progression of myeloma tumor burden and an efflux of calcium in the serum known as hypercalcemia., a prominent feature of late stage of the disease [112]. Activated BMSC and osteoblasts secrete also a relevant MM growth factor, Insulin-Growth Factor 1 (IGF1), which

promotes the growth, the survival and the migration of MM by activating MAPK and PI3/AKT signaling pathways. This activation of AKT provides a survival signal for plasma cells through the up-regulation the anti-apoptotic proteins Bcl-2 and Bcl-X_L and the down-regulation of the pro-apoptotic proteins BIM [113, 114]. The adhesion of MM to fibronectine protects tumors from DNA damaging agents (anthracylines and alkylating agents) [115]. Finally, within the bone marrow microenvironment, the adhesion of MM to plasmacytoid Dendritic cells triggers the secretion of growth factors that promote MM growth in paracrine manner and mediate immunodeficiency characteristic of MM [116].

In most patients, malignant plasma cells do not remain localized in the original site of tumor initiation, represented by primary plasmocytoma but they disseminate within the axial skeleton. The dissemination of MM involves the egress of MM into the blood stream and their homing back to the bone marrow, forming thus micrometastasis represented by the clinical condition of MGUS [117]. It has been shown that the loss of E-cadherin which is a key cell to cell adhesion molecule and the activation of epithelial mesenchymal transition associated to hypoxic conditions within the bone marrow niche may facilitate the dissemination /egress of MM cells and their homing to new sites within the bone marrow [118]. After a long period of latency, MGUS can progress to macrometastasis and manifest MM stage defined by the clinical symptoms including multiple lytic lesions, anemia, hypercalcemia and renal failure [119]. When MM cells no longer depend on the bone marrow microenvironment signals and acquire the anchorage independent growth, then they are able to proliferate outside the bone marrow microenvironment. At this stage, the number of circulating cells in the blood stream increases and lead to the development of plasma cell leukemia [120]. In other cases malignant plasma cells home to other organs (liver, gut, lungs) manifesting the extramedullary myeloma which corresponds to the most aggressive and drug resistant stage of the disease from which most HMCL are derived [121].

I.5 Treatment of MM

Multiple myeloma often progresses slowly and may not show any signs or symptoms. Patients with early stage of MM or MGUS and manifesting no symptoms may require only clinical surveillance. Symptomatic Myeloma patients, though, are immediately treated to reduce plasmacytoma burden, relieve symptoms and prevent complications [3]. Although Myeloma remains an incurable disease, remission can be achieved by chemotherapy, which destroys or controls only dividing cells. Chemotherapeutic agents that have been used successfully for the

treatment of MM include cyclophosphamid, doxorobucin, melphalan, etoposide and cisplatin [122]. Usually a combination of these drugs and other treatments such as targeted therapies and/or steroids (prednisone and dexamethasone) is more effective than the use of one single drug. Targeted therapies target, within malignant plasma cells, gene expression, protein turn over and signaling pathways that promote cell growth and survival as well as the tumor microenvironment. This type of drugs includes Thalidomide and lenalidomide, which are immune system modulators and have an antiangiogenic activity that may starve cancer cells [123].

Because myeloma cells produce many proteins, they might be vulnerable to the imbalance between protein synthesis and degradation. Bortezomib is a proteasome inhibitor that suppresses the ubiquitin proteasome system, which allows the accumulation of target proteins including the NF- κ B inhibitor keeping thus NF- κ B in inactive state. Accordingly, the inactivation of NF- κ B alters the activation of other signaling pathways that lead collectively to reduced cell proliferation and increased apoptosis of treated myeloma cells [124, 125]. In case of bone fracture by compression, localized high-energy radiation may alleviate bone pain, weaken Myeloma cells and prevent growth [126]. Because both high dose chemotherapy and radiotherapy may destroy hematopoietic stem cells in the bone marrow, autologous stem transplantation (ASCT) is performed in order to restore the hematopoietic system. Although high dose therapy combined with ASCT demonstrated superior response rates and progression free survival in comparison with conventional chemotherapy, the majority of MM patients experience disease relapse and progression [127-129]. Recurrent myeloma could be due either to reinfused cancer cells that contaminated the autologous graft before the treatment [130] or to drug resistance of some myeloma cells called myeloma stem cells that may possess a clonogenic growth to mediate tumor regrowth [131]. Recently, it has been evidenced that myeloma relapses from circulating clonotypic CD20+ CD27+ memory B cells with stem cell property [132]. It has been suggested that these cells might be target of the immunologically mediated Graft versus myeloma (GvM) effects mounted following allogeneic stem cell transplantation [133]. Much riskier than ASCT, allogeneic stem transplantation is limited only to very few patients because of Human Leukocyte histocompatibility. Moreover, the procedure has a greater risk of complication, including infections and graft versus host disease (GVHD) which is a potentially fatal condition while the donor's lymphocytes attack and destroy patient tissues. Despite morbidity, mortality and relapse risk, highest clinical and molecular remission rates are achieved following allogeneic stem cell transplantation. This is

mainly attributed to tumor free graft which may reduce relapse to 50% [134, 135] and to GvM effects mediated by donor lymphocytes that destroy myeloma cells still present in the recipient's bone marrow [136]. Whereas GvM induction is typically attributed to the anti-tumor activity of donor T cells [137], the protective effect of T cell depleted HLA-mismatched transplantation suggests a potential role for Natural killer cells [138]. In addition, the donor antibody response may have anti-tumor effects. Indeed, antibody responses to tumor-associated antigens have been observed in MM patients following allogeneic stem cell transplantation [139]. The application of the serologic analysis of recombinant cDNA expression libraries (SEREX) showed that these antibody responses targeted myeloma-associated antigens corresponding to extra and intra-cellular proteins [140], among them Cancer-testis antigens (CTA), particularly MAGE-C1/CT7, MAGE-C2/CT10 and MAGE-A3 which are commonly expressed in MM tumors [141]. Because frequent antibody responses against CTA develop following allogeneic stem cell transplantation and correlate with CD4+ and CD8+ T cell responses, it is thought that CTA may present natural targets for the GvM, which may exert an anti-tumor effect and reduce relapse risks [141-144]. Accordingly, CTA have received considerable attention as potential candidates for anti-cancer vaccine in order to enhance the efficiency of anti-myeloma immune response in the setting of allogeneic stem cell transplantation, prolong long-term remission and reduce relapse.

II. Cancer-testis antigens

II.1 The identification of CTA

CTA genes were originally identified by T-cell epitope labeling which is a DNA cloning methodology for defining targets of T cell recognition expressed by malignant cells. It was in 1991 that the first CTA, MAGE-A1 (Melanoma Associated Antigen-A1), was discovered by Van der brugen in a melanoma patient as an antigen presented by the major histocompatibility complex molecules of melanoma cells and being recognized by spontaneous autologous cytotoxic T cells [145]. This approach consists on the stimulation of PBMC of melanoma patients with autologous melanoma cell line. The clone tumor reactive cytolytic lymphocytes are then used to test the antigenic expression of cDNA library from melanoma cells divided in pools encoding antigens and transfected in Cos cells expressing the appropriate major histocompatibility complex molecules. Further analysis identified other closely related genes clustered at Xq28 consisting of MAGE-A family [146]. Subsequently, additional related clusters mapping at Xp21.3 and Xq26-27 corresponding respectively to MAGE-B [147] and

MAGE-C [148, 149] families were identified as well as other more distantly related clusters, MAGE-D and MAGE-L mapping at Xp11 and 15q, respectively [146]. From the same patient where MAGE-A1 was discovered, other CTA, non-structurally related to MAGE families, were discovered including B Melanoma antigens (BAGE) [150] mapping at juxtacentromeric regions of chromosome 9, 13, 18, 21 [151] and G antigen 1 (GAGE) [152] mapping at Xp11, being both recognized by autologous cytolytic T cell clones. Because of the difficulty, on a routine basis, in establishing patient derived tumor cell lines from other common cancers as well as the expansion of stable autologous tumor reactive cytolytic T cell clones, the majority of antigens discovered by the genetic approach were melanoma specific which limited the application of the genetic approach on other cancers [145]. The aforementioned SEREX approach, however, represented a more powerful and universally applicable approach to a variety of cancers. It consists on the immuno-screening of human testis cDNA libraries by using sera of cancer patients. This has led to the identification of antigens having elicited high titer IgG antibody responses in cancer patients [153] including the Synovial sarcoma (SSX) [154], the synaptonemal complex protein 1 (SCP1) [155] and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) which represents one of the most immunogenic CTA [156]. Because both approaches, whether genetic or serological, illustrated well the antigenic properties of CTA and because further analysis showed that these tumor-associated antigens are faithfully expressed in cancers but not in healthy tissue except testis, which is an immune privileged site [157], CTA presented attractive targets for cancer immunotherapy. Therefore extensive efforts have been conducted into the development of other tools suitable for the identification of other immunogenic CTA, among them the representative differential expression analysis of cDNA for the identification of genes differentially expressed in tumors [158] and the reverse immunology approach for the characterization of the immunogenic properties of the encoding proteins. In this approach, candidate genes are selected based on their expression restricted to tumors and their putative antigenic determinants are predicted by bioinformatic algorithm based on proteasomal cleavage sites as well as their binding to major histocompatibility complex. The immunogenic peptides are afterwards assessed experimentally *in vitro* by their recognition by cytolytic T clones and finally validated by tumor cell recognition [159]. This technique led to the identification of MAGE-1[160], MAGE-2 [161], MAGE-3 [162] and SSX-2 [163]. (Table 1)

Table 1. List of selected known X and Non-X Cancer-testis antigens

	Cancer Testis antigen family	Number of genes	Chromosome	Original identification method	Spontaneous immunity in cancer patients	References
	MAGE-A	15	Xq28	CTL epitope cloning	Cellular and humoral	[146]
	MAGE-B	17	Xp21	Exon trapping	Cellular	[147]
	GAGE	8	Xp11.4	CTL epitope cloning	Cellular	[164]
X-CTA	SSX-2	5	Xp11.2	SEREX	Cellular and humoral	[154]
	NY-ESO-1	3	Xq28	SEREX	Cellular and humoral	[156]
	CT7/MAGE-C1	7	Xq26-27	SEREX	Humoral	[148]
	CT10/MAGE-C2	1	Xq27	RDA	Humoral	[149]
	HOM-TES-85	1	Xq24	SEREX	Humoral	[165]
	BAGE	5	4,13	CTL epitope cloning	Cellular	[150]
Non-X	SCP-1	3	1p12-p13	SEREX	Humoral	[155]
CTA	OY-TES-1	1	12p13	SEREX	Humoral	[166]
	cTAGE-1	1	18p11.2	SEREX	Humoral	[167]

MAGE, Melanoma antigen; GAGE, G antigen 1; SSX, synovial sarcoma X; NY-ESO-1, New York oesophageal squamous cell carcinoma 1; BAGE, B Melanoma antigen; SCP-1, synaptonrml complex protein 1; RDA, representional differential analysis. (adapted from [168])

II.2 CTA expression in normal and cancer tissues

To date, about 70 families of CTA, have been identified accounting over 140 members [169]. Based on chromosome mapping, CTA consist broadly of two groups including X-CTA which are encoded by genes mapping to chromosome X, organized in well-defined clusters [170, 171] and Non-X CTA which are encoded by genes distributed throughout the genome, organized as single copy genes [155]. The analysis of gene expression shows that CTA gene expression is epigenetically silenced in healthy tissue except germ cell lines including testis and occasionally placental trophoblast [172, 173]. Adult testis presents a diverse pattern of CTA expression. While X-CTA are frequently expressed in mitotically proliferating germ spermatogonial cells, Non-X CTA are predominantly expressed in later stages of germ line differentiation that coincide with meiosis such as spermatocytes. It should be noted, however, that a low mRNA expression less than 1% of some CTA genes were detected in somatic tissues, including pancreas, liver and spleen [174]. During neoplastic transformation, CTA expression is reactivated mainly due to genome-wide hypomethylation frequently observed in cancer [175] and this expression seems to vary greatly between tumors of different histological types. The highest frequencies have been reported in solid tumors particularly

melanoma, lung cancer, while moderate frequencies were observed in breast and prostate cancer. A weak CTA expression, on the other hand, was found in renal cancer, colon cancer and hematological malignancies [172]. Even among CTA members, the frequency of expression varies considerably. While MAGE-A1, MAGE-A3, SSX2, SSX4 and NY-ESO-1 are the most frequently expressed antigens, BAGE, GAGE-1 and SCP-1 are rarely expressed [168]. One important feature of CTA expression, particularly X-CT, is that they are often concomitantly expressed in the same tumor [176]. The co-expression, particularly, of X-CT, seems to be a part of coordinated program because in tumor expressing X-CT, MAGE-A3 is always expressed irrespective of any other X-CT, while NY-ESO-1 is never expressed in the absence of MAGE-A3 [172]. Unlike other Tumor-associated antigens, which display a homogenous pattern of protein expression in a single tumor, CTA show often a heterogeneous antigen expression. For instance, immunohistochemical staining for NY-ESO-1 and MAGE-A1 protein expression in non-small cancer shows 5 to 50% positive cells which illustrates the intratumoral heterogeneity of CTA expression in tumors [170, 177]. Only a singular homogeneous expression pattern for NY-ESO-1 was in synovial sarcoma [178]. Although the reasons for this pattern of expression are not yet understood, the finding, that melanoma stem cells are expressing homogeneously MAGE-A1, suggests that CTA may be markers for cancer stem cells within tumors which are thought to be the ultimate targets for efficient cancer treatments [179].

II.3 Regulation of CTA expression

All CTA genes have methylated promoters in somatic tissues and their expression is activated by demethylation during spermatogenesis [180]. In tumors, promoter hypomethylation represents a key epigenetic event that regulates CTA expression, because the treatment of melanoma cell line with a DNA demethylating agent, 5-aza-2'-deoxycytidine (5-AZA-CdR) activated de novo CTA expression [181]. Yet, the weak expression of CTA in colon cancer, which displays a hypomethylated genome, suggests the involvement of a secondary epigenetic mechanism [182]. The treatment of Myeloma cells with IL-7 and granulocyte growth factor up-regulates the expression the X-CTA gene SPAN-Xb but only in the presence of demethylated promoter. These observations suggest that some specific cytokine may operate as a secondary mechanism for the regulation of CTA expression [183]. Additionally, an up-regulated transcriptional activity of both methylated and unmethylated MAGE-A2 and MAGE-A12 promoters was observed following histone deacetylase inhibitor treatment of

tumor cells suggesting that histone modifications may also play a role in the epigenetic regulation of CTA [184].

II.4 Correlation between CTA expression and tumor progression

Clinical correlations demonstrated that the expression of CTA, particularly members of MAGE class I proteins, is associated with disease progression, higher grade and metastatic tumors, poor prognosis and resistance to chemotherapeutic agents, suggesting a potential involvement of MAGE class I and other CTA in promoting cell survival by modulating proliferation, apoptosis and metastasis [185-188] (Table 2). Because of their expression restricted to tumors, associated with disease progression and metastatic potential as well as their immunogenicity, CTA become extremely attractive as a potential tumor markers for diagnosis, prognosis and as targets for cancer vaccine. Therefore, an understanding of their biological contribution to the malignant phenotype and the process of tumorigenesis whether initiation and/or progression is needed.

Table 2. Correlation of Cancer -testis protein expression with clinicopathological features and prognosis

Tumor type	Cancer Testis antigen	Association	References
Melanoma	MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4	Tumor thickness and metastasis	[186]
Non-small-cell lung cancer	MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, MAGE-C1	Advanced tumor type, nodal and pathologic stages as well as pleural invasion	[176]
Pancreatic cancer	MAGE-A3	Poor survival	[189]
Hepatocellular carcinoma	MAGE-C1	Reduced overall survival	[190]
Multiple myeloma	MAGE-A1, MAGE-A3, MAGE-A4, MAGE-C1	Stage and risk status of disease	[191-193]
Serous ovarian carcinomas	MAGE-A4	Inverse correlation between expression and patient survival	[194]
Melanoma	NY-ESO-1	Thicker primary lesions and a higher frequency of metastatic disease	[195]

(Adapted from [174])

II.5 The function of CTA

II.5.1 Function of Non-X CTA

In germ cells, most of Non-X CTA have known roles in spermatogenesis and fertilization processes. Among them SCP-1 and SP11, which are selectively expressed in spermatocytes during the first meiotic prophase, are integral components of the synaptonemal complex and regulates chromosome homologues pairing during the first meiotic division [196, 197]. The

brother of the regulator of imprinted site (BORIS) is another Non-X CTA member and a transcription factor, expressed in spermatocytes, that is thought to regulate the epigenetic reprogramming occurring during spermatogenesis [198], the demethylation of CTA gene promoters and the activation of their expression in tumors [199]. Later during germ cell differentiation, the Non-X CTA TPX-1 is expressed in spermatocytes, mediates binding of the spermatogenic cells to Sertoli cells. Moreover, being component of the sperm acrosome, TPX-1 as well as the metalloproteinase ADAM are thought to be relevant in sperm-oocyte interaction [200]. SMEG1, which is the predominant compound of the seminal plasma, was described to be involved in the regulation of spermatozoon mobility [201]. Although their role in cancer is not well understood, it is likely that the activation of Non-X CT expression in cancer may lead to the activation of the gametogenic programs that may contribute to the shared features between germ and tumor cells including demethylation, down-regulation of the major histocompatibility complex (immune escape), immortalization, induction of meiosis (aneuploidy and genomic instability), migration and invasion (metastasis) [172],

II.5.2 Function of X-CTA

The expression of X-CTA particularly members of the large MAGE family in spermatogonia, primary spermatocytes and round spermatids but not in elongating spermatids and spermatozoa suggests that MAGE proteins may promote the proliferation of spermatogonia by regulating cell cycle progression and may be involved in the biological events of meiotic division and early spermiogenesis [202]. Some evidences indicate that MAGE proteins may promote the survival of male germ lines by suppressing apoptosis. Indeed, it has been shown that the hypomethylated state of MAGE gene promoters in germ cells is maintained by the c-Kit oncogene and that the activation of the KIT receptor tyrosine kinase promotes MAGE gene expression. Interestingly, the loss of MAGE expression because of Kit partial loss of function in mice induces infertility, increases germ cell apoptosis and decreases germ cell viability. These observations indicate that MAGE proteins may support the self-renewal of germ stem cells and protect germ cells from apoptosis during meiosis. Besides the anti-apoptotic role of MAGE proteins, it has been shown that MAGE-A1 may act as a transcriptional repressor of genes required for differentiation, as it is expressed in spermatogonia but not during later developmental stages. MAGE-A1 was found to disrupt NOTCH1 signalling pathway by binding to SKIP and recruiting histone deacetylase. It is, therefore, hypothesised that a similar function may contribute to cancer dedifferentiation.

Although their biological function within germ cells is still unclear, ample evidences show that MAGE proteins and other X-CTA may play a role in tumorigenesis. Indeed, clinical correlations indicated that MAGE and other X-CTA expression in tumors contributes to disease progression, aggressiveness and resistance to chemotherapy. It was found that the expression of at least one of these genes MAGE-A1, MAGE-A2 or MAGE-A3 may confer resistance to Tumor necrosis factor cytotoxicity [203]. Additionally, the expression of MAGE-A2 and MAGE-A6 confers resistance to the chemotherapeutic agents Paclitaxel and Doxorubicin, typically observed in aggressive cancers [204] suggesting that MAGE proteins may alter the apoptotic response induced by DNA-damaging agents. Moreover, the intriguing observation that mesenchymal stem cells, CD34+ hematopoietic stem cells and spermatogonia stem cells express a wide range of MAGE and other X-CTA points to common properties or mechanisms in these cells [172, 205, 206]. One likely link is the self-renewal/the unlimited proliferation and the resistance to apoptosis in response to DNA damage, which is thought to be controlled by MAGE proteins conferring thus drug resistance in cancer.

Melanoma-associated antigens (MAGE) belong to an extensive family of proteins that includes more than 60 genes sharing in common a conserved stretch of ≈ 200 amino acids called the MAGE homology domain (MHD) [146]. MAGE-like genes are found in non-mammalian species including *Drosophila melanogaster*, *Danio rerio* (Zebrafish) and in some flowering plants such as *Arabidopsis thaliana*, but not in *Caenorhabditis elegans*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* which may indicate evolutionarily conserved functions for MAGE genes [146]. MAGE family consists of two subfamilies MAGE class I and II. MAGE class I gene family, formed by MAGE-A, -B and -C genes, is clustered on chromosome X and encodes for tumor specific proteins belonging to the group of CTA [172, 173]. Unlike MAGE class I, class II family members represented by NECDIN, MAGE-D and MAGE-G, do not have defined chromosomal cluster and are not specifically related to cancer as they are ubiquitously expressed in a variety of somatic tissues and are implicated in neuronal differentiation and apoptosis [207-211]. For instance, NECDIN deletion is associated with Prada-Willi syndrome (a neurobehavioral disorder and the genetic cause of life threatening obesity in children). A tumor suppressor role has been proposed for NECDIN because of its down-regulation in tumors and its involvement in the repression of p53 and E2F1 transcriptional activity and in sustaining hematopoietic stem cell quiescence [212].

Emerging data support a role for MAGE class I in the regulation of the tumor suppressor p53 which is a transcription factor that controls genome integrity and can be induced by DNA damage and oncogenic stimulation leading to the activation of a selective gene expression program mainly involved in cell cycle arrest, senescence or apoptosis [213, 214]. In the absence of genotoxic or oncogenic stress, MAGE class I proteins suppress p53 activity by blocking the DNA binding interface of the core domain which impairs p53 DNA binding to its targets promoters [215] and by targeting p53 for ubiquitin-dependant proteasome degradation due to MAGE/RING domain protein interaction [216]. Multiple MAGE proteins including MAGE-A3, MAGE-C2/CT10 and murine mageb were shown to form complexes with KAP-1 which is a co-repressor of p53 and E3 ubiquitin ligase enhancing thus the polyubiquitination of p53 and consequently its degradation in proteasome dependent manner [216]. Under stress conditions, induced p53 is further suppressed by deacetylation of p53 and DNA surrounding p53 response elements, through the enhancement of histone deacetylases (HDAC) recruitment which may impair p53 transcriptional activity, suppress DNA damage mediated p53-dependent apoptosis and enhance resistance to etoposide treatment [217, 218]. In addition to the suppression of p53-dependent apoptotic response, HDAC-dependent mechanism of MAGE proteins may impair cellular senescence which is an important barrier against oncogene induced cell transformation by impairing the promyelocytic leukemia tumor suppressor (PML) sumoylation which affects p53 acetylation and transcriptional activation [219]. These evidences highlight the relevance of MAGE/p53 interaction in tumor initiation, progression and resistance to apoptosis. Downstream p53 apoptotic pathway, MAGE-A3 may enhance cancer cell survival by disrupting caspase 12 mediated apoptosis to drug induced insults through the blocking of pro-caspase 12 activation [220].

Besides the suppression of p53-dependent apoptosis, it was shown that MAGE-11 might facilitate prostate cancer progression by enhancing androgen receptor-dependent growth. MAGE-11 seems to stabilize the androgen receptor in the absence of hormone thereby promoting the recruitment of the steroid co-activator (SRC)/p160 and the subsequent activation of the androgen receptor transcriptional activity. This results into the amplification of the androgen signalling pathway often observed in recurrent prostate cancer following androgen deprivation therapy suggesting that MAGE-11 may promote hormone refractory prostate cancer [221].

Similarly to MAGE, GAGE7C expression in tumors confers resistance to apoptosis induced by $\text{INF}\gamma$, FAS, Taxol and γ -irradiation [222]. Consistent with these findings, recent

investigations have shown that GAGE may impair the apoptotic response by suppressing the transcriptional activity of a key player in the INF γ signalling pathway, the interferon regulatory factor (IRF1) which leads to a decrease in caspase-1 and caspase-7 [223].

SSX proteins are thought to be transcription factors because they bear a domain sharing high sequence homology with Kruppel-associated Box (KRAB) of the transcriptional repressors zinc finger proteins. SSX1 and SSX2 were originally discovered as fusion partner with SYT gene as a result of the chromosomal translocation (X;18) (p11.2;q11.2) in synovial sarcoma [224]. The resulting fusion protein SS18-SSX down-regulates COM1 in synovial sarcoma tissue and cell lines, which is a regulator of cell proliferation and the single downstream target known so far [225].

II.5 Immunogenicity of CTA

Because of their ability to induce spontaneously a humoral and/or cellular immune response in cancer patients as illustrated by T-cell cloning and SEREX approaches, their restricted expression to tumors and their potential contribution in the malignant phenotype, CTA are thought to be ideal targets for cancer immunotherapy. Yet, a better characterization of the anti-tumor immune response is required in order to establish efficient immunotherapy strategies. Indeed, to date a spontaneous and coordinated humoral and cellular immune responses have been demonstrated against several CTA such as MAGE-A1, MAGE-A3, SSX-2 and NY-ESO-1 as well as their Human leukocyte antigen (HLA) restricted T cell epitopes and their corresponding HLA antigen alleles (Table 3). Several studies demonstrated that NY-ESO-1 is one of the most immunogenic CTA because of frequent antibody responses detected in 10 to 86% of cancer patients expressing NY-ESO-1 including thyroid cancers, lung cancers, ovarian cancers, breast cancers, bladder cancers, esophageal cancers and melanomas [202]. The titer of the NY-ESO-1 antibodies increases with disease progression and decreases upon disease regression or removal. Furthermore, the analysis of T cell response showed that more than 90% of patients, developing NY-ESO-1 antibody response, evidenced a spontaneous NY-ESO-1 CD8⁺ T cell response [226]. Similarly to NY-ESO-1, a coordinated humoral and cellular immune response has been observed for MAGE-A and SSX proteins but at lower level. Additionally an antibody immune response has been reported for SCP-1 in 50% of breast cancer patient, MAGE-A1 in 36% in melanoma patients, cTAGE in 33% cutaneous T-cell lymphoma and SSX-2 in 18% of melanoma patients [168].

Table 3. Immunogenicity of NY-ESO-1, MAGE-A1, MAGE-A3 and SSX-2

Cancer Testis antigens	mRNA expression frequency	Frequency of serum antibody in cancer patients	T cell epitopes			
			CD8+		CD4+	
NY-ESO-1	34% Melanoma	9% Melanoma	A2		DR4	
	29% Ovarian Cancer	12% Ovarian Cancer	A31			
	16% Lung Cancer	4% Lung Cancer	Cw3			
	24% Breast Cancer	8% Breast Cancer	Cw6			
MAGE-A1	16% Melanoma	1% Melanoma	A1	B35	Cw2	DR13
	28% Ovarian Cancer	3% Ovarian Cancer	A3	B7	Cw3	DR15
	49% Lung Cancer	4% Lung Cancer	A24	B53	Cw16	
	18% Breast Cancer	0 Breast Cancer	A28			
MAGE-A3	36% Melanoma	2% Melanoma	A1	B35		DR4
	17% Ovarian Cancer	0 Ovarian Cancer	A2	B37		DR7
	47% Lung Cancer	0 Lung Cancer	A2	B40		DR11
	11% Breast Cancer	0 Breast Cancer	A24	B44		DR13
			A24	B52		
SSX-2	35% Melanoma	1% Melanoma	A2		Unknown	
	0% Ovarian Cancer	0 Ovarian Cancer				
	17% Lung Cancer	0 Lung Cancer				
	7% Breast Cancer	0 Breast Cancer				

From the peptide database of T-cell defined tumor antigens. (adapted from [168])

<http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>

II.6 Cancer vaccine trials targeting CTA

The observation that chronically immunosuppressed patients have an increased risk of cancer [227] and that rare spontaneous tumor regressions are often preceded by infectious episodes [228] led to the development of the concept of tumor immune surveillance. In order to use own body response to fight cancer, a number of cancer immunotherapeutic strategies have been established consisting of two category groups passive and active cancer immunotherapy. The passive approaches rely on the administration of substances that modulate transiently the immune system such as cytokines (IL-2 or interferon α) or antibodies. In contrast, the active approaches such as cancer vaccines can provide immunological memory by stimulating the patient's immune system and particularly cytotoxic T cells which are the key mediator of the cellular immunity, able to recognize specifically the tumor-associated antigens expressing neoplastic cells, eradicate them and persist even after the treatment has stopped [229].

Due to their expression restricted to tumors and their immunogenicity, CTA appear to be ideal targets for tumor vaccination. Currently, this new therapy is a growing field that extends to many cancers and aims to develop immunization protocols that induce a more effective antitumor immune response consisting of uniformly memory effectors CD8 + and CD4 + T cells with a very fine antigen specificity and ability to migrate to the tumor site. Accordingly, several clinical trials targeting MAGE-A3 and NY-ESO-1 are ongoing or have been completed. For instance, clinical trials in metastatic melanoma using peptide based vaccines,

for both antigens, demonstrated a significant tumor regression. Yet MAGE-A3 vaccination showed a poor immunological outcome in comparison to NY-ESO-1 peptide based vaccine, as no evidence of MAGE-A3 cytotoxic T cell response was found even in patients with complete tumor regression [230, 231]. Going forward, significant expansion of MAGE-A3 CTL was achieved while vaccinating metastatic melanoma patients with Monocytes derived Dendritic cells loaded with HLA-A1 restricted MAGE-A3 peptide. The induced immunological response led to a tumor regression and T cell infiltration in regressing metastasis. Additional clinical trials in patients with melanoma, non-small lung cancer, bladder and gastro-intestinal cancer immunized with MAGE-A pulsed Dendritic cells showed similar immunological and clinical responses indicating that the active immunization with MAGE-A may change the course of MAGE-A expressing tumors [169]. Additionally, recombinant full-length protein immunization has been proposed because it can induce a broader spectrum of CD4+ and CD8+ T cell responses against multiple epitopes, unrestricted of the HLA type and can therefore be applied to a larger number of cancer patients expressing MAGE-A3. The His-tag MAGE-A3 fused to the N-terminus protein D domain of Haemophilus influenzae was the first full-length recombinant protein based vaccine used for the immunization of advanced melanoma patients. The results of this study showed, however, overall, weak immunological and mixed clinical responses [232]. Subsequently, given the low tumor response rate and the mild toxicity, phase II clinical trials have been launched in order to evaluate the efficiency of the anti-tumor response following the immunization of patients with unresectable and progressive metastatic cutaneous melanoma with MAGE-A3 protein combined with AS15 and AS02B adjuvant systems. The results reported an objective response and long term disease stabilization as well as an induction of MAGE-A3 specific antibody and CD4+T cell responses [233]. Given these promising findings in melanoma, phase II clinical trial was conducted in patients with non-small lung cancer (NSCLC) and an improvement of disease free survival was observed in 33% of vaccinated patients in comparison with placebo [234]. Further investigations have shown that the clinical efficiency of MAGE-A3 antigen specific cancer immunotherapy is closely related to a favorable specific gene signature [235]. Given these preliminary data, a randomized, double blind phase III study known as MAGRIT (MAGE-A3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy), enrolling 2270 NSCLC patients, has been initiated in 2007 which is the largest-ever trial in the adjuvant treatment of NSCLC and aims to investigate the efficacy of MAGE-A3 ASCI in preventing cancer relapse after tumor resection, in patients expressing MAGE-A3 [236].

As far as NY-ESO-1, phase I clinical trial using His-tagged recombinant NY-ESO-1 with ISOMATRIX adjuvant (saponin-based adjuvant) was conducted for the vaccination of melanoma patients. Although the vaccination induced an integrated CD4+ and CD8+ anti-tumor responses against a broad range of NY-ESO-1 epitopes, a favorable clinical response was observed in minimal residual disease but not in advanced melanoma patients, which was attributed to the immunosuppression of regulatory T cells [237, 238]. Consistently with these observations, a more recent study showed that the treatment of metastatic melanoma patients with CTL-A monoclonal antibody (ipilimumab) combined with NY-ESO-1 vaccine could synergistically enhance the clinical effect. It was found that this effect was due to the ability of CTL-A monoclonal antibody to enhance the polyfunctional NY-ESO-1-specific-T cell responses which may help eradicate metastatic cancer cells [239].

Despite the promising outcome of CTA based vaccine clinical trials, the heterogeneity of CTA expression in cancer cells may limit the beneficial effect of the anti-tumor response because of the emergence of CTA negative resistant clones that may escape the established CTA immune surveillance. Because the intratumoral heterogeneity of CTA expression is mainly dependent on the extent of their promoter methylation, the epigenetic modulation of CTA expression by DNA demethylation agents such as 5-AZA-CdR in combination with CTA vaccine may contribute to homogenous targeting of the neoplastic cells by CTA cytotoxic T lymphocytes [240]. Furthermore, 5-AZA-CdR is also characterized by an immunomodulatory activity consisting of a persistent up-regulation of different molecules involved in antigen presentation such as HLA class I antigens and co-stimulatory molecules, which may improve the recognition of CTA by CTA specific CTL [241]. Along the line, recent phase I clinical trial, investigating systemic 5-AZA-CdR in patients with thoracic malignancies, demonstrated de novo expression of NY-ESO-1 and MAGE-A3 in almost 37% of treated cancer patients, among them 37% of patients induced a humoral response against NY-ESO-1 suggesting that the novo CTA induction might be recognized by the immune system [242].

III. Cancer-testis antigens in MM

III.1 CTA expression in MM

Among hematological malignancies, MM is probably the one tumor type with the richest expression of CTA. A frequent expression of CTA has been described in MGUS, MM

primary specimen of patients and HMCL, with MAGE class I family members being the most commonly expressed CTA specially in MM patients with stage III including MAGE-A3, MAGE-C1/CT7 in 80 to 100% of cases [243]. While NY-ESO-1 and MAGE-C2/CT10 are expressed in up to 60% of MM patients [141, 142], the frequency of other CTA, such as SSX-1, SSX-4, SSX-5, SSX-2, BAGE, ADAM [141], GAGE-1 [244], SCP-1 [245], Sp17 [246], SLLP-1 [247], SPAN-Xbox [248] and SEMG1 [249] is typically between 6 to 40%. Overall, CTA are often co-expressed heterogeneously in MM tumors and even within a single tumor [250]. CTA expression was detected across the entire spectrum of malignant gammopathies, from medullary plasmacytoma to MM and extra-medullary plasmacytoma. Because of an increased frequency of expression during disease progression, many investigations support the notion that CTA expression might be linked to clonal evolution [193]. Higher levels of CTA expression particularly MAGE class I family proteins were found to be correlated with elevated plasma-cell proliferation, advanced stages of the disease and cytogenetic abnormalities (chromosome 13q deletion and 1q21 gain) [141, 243, 250-252]. Consistently, a more recent study has shown that a high expression of a large group of CTA (MAGE, SXX, GAGE, CTAG) is associated to mitosis/proliferation gene expression and defines the molecular signature of the most aggressive variant among hyperdiploid MM cytogenetic group [49]. In addition to their prognostic value, CTA represent a potential marker for minimal residual disease after stem cell transplantation and their expression has been strongly linked to the resistance to chemotherapeutic agents and to shorter survival [250]. Accordingly, the common expression, correlation with proliferation and non-favorable clinical outcome suggest that MAGE class I and other CTA may confer a survival advantage on myeloma cells and represent ideal targets for the eradication of minimal residual disease.

III.2 The immunogenicity of CTA in MM

It has been shown that CTA are not only expressed by malignant plasma cells but they are immunogenic and present natural targets for the GvM following allogeneic stem cell transplantation, which is thought to exert an anti-tumor effect and reduce relapse risks [141-144]. Indeed, MM patients were found to develop high titer antibody responses against SPAN-Xb [248], SLLP-1[247], NY-ESO-1[142], MAGE-C1[253] which may imply the involvement of CD4+ T cell response. More importantly, the CTA specific B cell responses were coordinated with spontaneous CTA specific cytotoxic T cell responses, as CTA specific CD8+ T cells were detected in the peripheral blood of MM patients and when successfully expanded they were able to kill autologous primary MM cells expressing the respective CTA

[142, 254]. Accordingly, CTA have received considerable attention as potential candidates for T cell and possibly antibody-based immunotherapy of myeloma in order to enhance the efficiency of anti-myeloma immune response in the setting of allogeneic stem cell transplantation without inducing the GVHD which may provide a memory immune surveillance and consequently prolong long-term remission and reduce relapse often failed to be achieved with conventional treatments in the majority of MM patients.

III.3 Immunotherapy targeting CTA in MM

Early attempts to apply cancer immunotherapy for the management of MM targeted the idiotypic (Id) protein secreted by myeloma clones by immunizing early stage and early relapse MM patients with Id-pulsed Dendritic cells. Although the vaccination induced a coordinated humoral and cellular anti-id immune response in some patients, the results were disappointing because of weak anti-idiotypic immune response to control the growth of myeloma cells *in vivo* [255]. In another phase I clinical trial, relapsed MM patients were vaccinated with sp17-pulsed dendritic cells after allogeneic stem cell transplantation. The vaccination induced *in vivo* an anti-tumor immune response and led to 90% drop in the serum paraprotein level in addition to GVHD effects likely due to the expression of Sp17 in normal tissue [256]. In a more recent study, an adoptive transfer of PBMC from a syngeneic healthy donor, that were primed with His-tag MAGE-A3 fused to the N-terminus protein D domain of Haemophilus influenza and AS020 adjuvant system, to a stage III MM patient were performed following melphalan-based peripheral blood stem cell transplant. While MAGE-A3 recombinant protein induced, in the donor, an MAGE-A3 antibody response, a strong MAGE-A3 antibody, cytotoxic T cell and regulatory T cell responses were induced in the recipient that lasted 1 year following immunization and was associated with complete remission for 2.5 years [257]. Currently, two clinical trials of immunotherapy in MM are ongoing: (i) MAGE-A3 protein in combination with AS15 adjuvant system for MM patients undergoing autologous stem cell transplant (Ludwig Institute for Cancer Research/GlaxoSmithKline) (ii) MAGE-A3 and NY-ESO-1 immunotherapy in combination with DTPACE chemotherapy (Myeloma Institute for Research & Therapy, University of Arkansas)[202].

III.4 MAGE proteins regulators of p53 in MM

In contrast to chemotherapy, which is toxic and non-specific, cancer vaccine is usually less toxic and highly specific. Nonetheless, cancer vaccine is considered efficient in bringing a

clinical benefit only when it eradicates or at least control the tumor. Therefore it is important to target CTA whose the activation is relevant for tumor progression rather than being the neutral side effect of a global demethylation process that activates other genes involved in tumor progression. Recent functional genomic studies using transient or stable partial loss of function have reported an anti-apoptotic properties of MAGE proteins in MM which may promote the survival of malignant plasma cells and confer resistance to apoptosis induced by chemotherapeutic drugs [258, 259]. A more recent study demonstrated that MAGE-A may promote the survival of proliferating myeloma cells by repressing p53-dependent and independent apoptotic pathway through the down-regulation of the pro-apoptotic proteins BAX and BAK and the up-regulation of surviving (BIRC5) which is a regulator of the mitotic spindle checkpoint [252]. These findings highlight the relevance of MAGE/p53 interaction in MM being among the few tumors that do not frequently mutate p53 and rarely if ever p53 mutations or deletions occur late in the pathogenesis during advanced or extra-medullary disease [66]. Although inactivating mutations of p53 may confer resistance to the apoptotic triggers, some tumors that do not frequently mutate p53 such, as the case of MM, overexpress a range of proteins, that target and impair wild-type p53 function [260, 261]. Importantly, the observation that the selective activation of p53 apoptotic pathway with the small-molecule antagonists of MDM2 (nutlin-3) induced apoptosis in 90% of primary myeloma tumors, suggests that p53 apoptotic pathway remains functional [262]. Therefore, non-genotoxic activation of p53-dependent apoptotic pathway by suppressing p53 protein inhibitors, such as MAGE proteins, might be an attractive therapeutic strategy for this disease.

IV. Cancer-testis antigen MAGE-C2/CT10

IV.1 MAGE-C2/CT10 expression in cancer

MAGE-C2 is a member of MAGE-C family that was originally discovered in a specific cDNA library established from human melanoma cell line, by Representational differential analysis, a technique of subtractive hybridizing which has previously led to the identification of a closely related gene MAGE-C1/CT7 [148]. MAGE-C2 gene maps to MAGE-C family cluster at Xq27.13, shares with MAGE-C1/CT7 70% and 56% nucleotide and amino-acid similarities, respectively, and encodes for 2 slicing variants [263]. As seen for the other CTA, MAGE-C2 is expressed in healthy foetal and adult germ cells in the seminiferous tubules and extends to purkinje cells in the cerebellum [264, 265]. In adult testis, while MAGE-C2 is weekly expressed in spermatogonia, primary spermatocytes show a strong MAGE-C2

expression and in both cell types MAGE-C2 has a nuclear sub-cellular localization. At later stages of maturation, MAGE-C2 expression gradually decreases and becomes undetectable in mature spermatids [265, 266]. Yet, the biological significance of MAGE-C2 expression in healthy germ cells is poorly understood.

Like other MAGE family members, MAGE-C2 is frequently expressed in a wide range of solid tumors such as Hepatocellular carcinoma (34%-48%) [190, 267], primary and metastatic melanomas (20%,40%) [268], advanced high-grade urothelial carcinomas of the urinary bladder (20%) [269], head and neck cancers (20%)[149], colorectal cancers (5%) [270], metastatic and castration resistant prostate cancers (16%-17%) [271]. Furthermore, MAGE-C2 expression often correlated with advanced stages of Larynx squamous cell carcinoma [272], poor survival in advanced high-grade urothelial carcinomas of the urinary bladder, presented an indicator of lymph node metastasis in melanoma [268] and a biochemical recurrence in prostate cancer after radical prostatectomy [271].

IV.2 MAGE-C2/KAP1 complex suppresses p53 function

This frequent expression of MAGE-C2 in various tumors as well as the non-favourable clinical correlations supports a role for MAGE-C2 in promoting the tumorigenic process. An anti-apoptotic role for MAGE-C2 has been proposed by yang and colleagues, in two studies, demonstrating that the transient depletion of MAGE-C2 expression in melanoma and mast cells slowed the proliferation and induced p53 dependent apoptosis *in vitro* and *in vivo* [218, 273]. More recently, a biochemical analysis revealed that MAGE-C2 may interfere with p53-dependent apoptosis by enhancing the E3 ubiquitin ligase activity of Ring domain protein, known as a co-repressor for p53, the KAP1 (TRIM-28), which targets p53 for polyubiquitination and proteasome degradation [216] (Figure 2). Besides the regulation of p53-dependent apoptosis, KAP1 acts as scaffold protein that mediates gene silencing through the formation of heterochromatin [274] and following DNA damage, KAP1 is rapidly phosphorylated by ATM kinase, a key DNA damage responsive Kinase required for the homologous recombination double strand break repair. ATM mediated phosphorylation of KAP1 causes a local chromatin decondensation which facilitates the repair of damaged DNA [275]. Recently, it has been shown that MAGE-C2/KAP1 interaction may enhance ATM-dependent phosphorylation of KAP1 in response to doxorubicin-induced DNA damage which suggests a role of MAGE-C2 in the DNA damage repair response [276] and that MAGE-C2 may confer a survival advantage to cancer cells by suppressing p53-dependent apoptosis in

response to DNA damage. Accordingly, these findings highlight the relevance of MAGE-C2 mediated p53 inactivation in tumorigenesis and particularly in tumors that do not frequently mutate p53 such as MM [66].

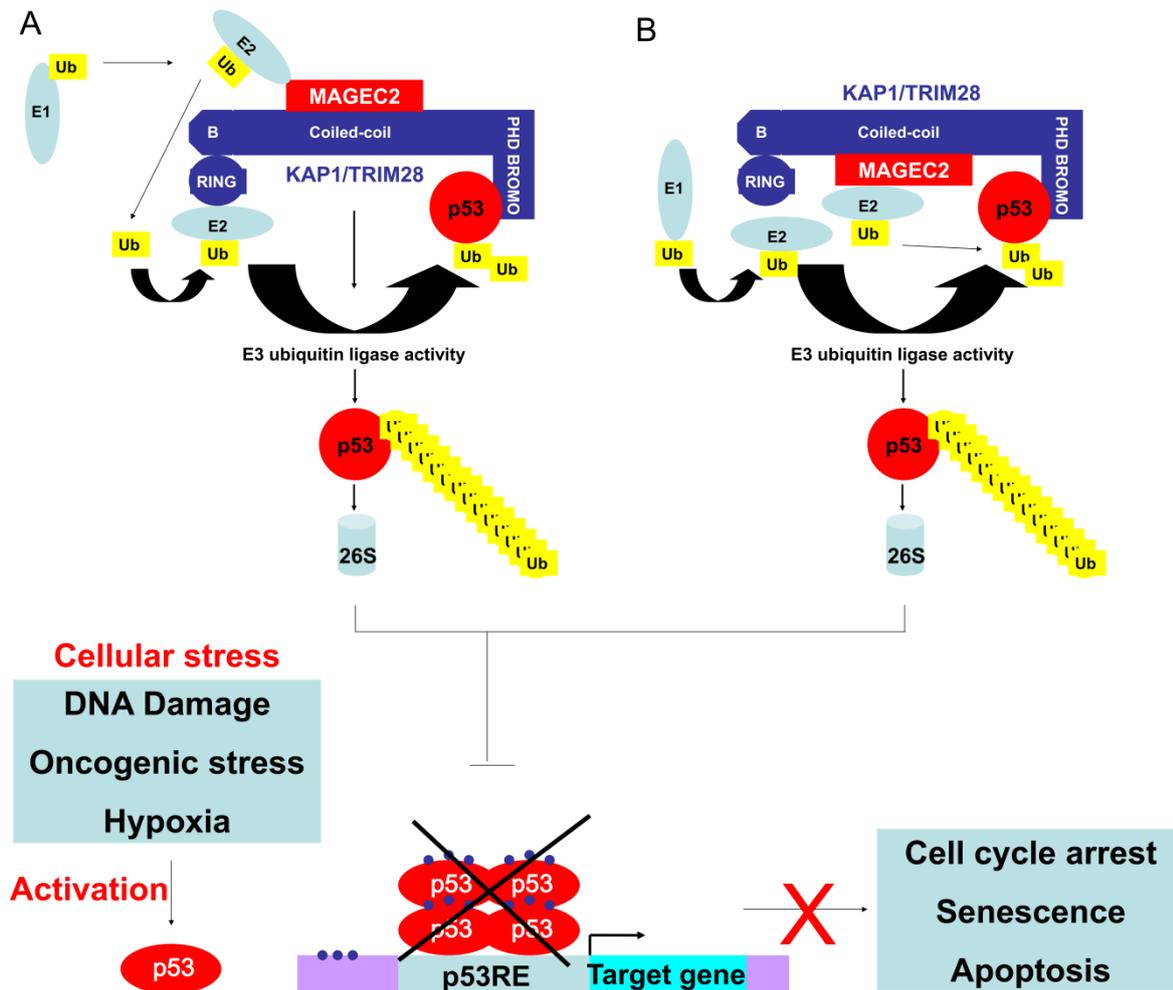


Figure 2: Models for the mechanism of MAGE-C2/CT10 mediated activation of the E3 ligase activity of KAP1/TRIM28 and its impact on p53 function. (A) In the first model, MAGE-C2 may facilitate the on-site recharging of the E2 conjugating enzyme. **(B)** In the second model, MAGE-C2 may promote the sequential assembly of the polyubiquitin chain on the substrate. As a consequence, MAGE-C2/KAP1 complex enhances the proteasome-dependent reduction of p53 level. Under stress conditions, p53 accumulates, undergoes post-translational modifications (acetylation and phosphorylation (blue spots)), tetramerizes and binds to p53 responsive elements which translates the cellular stress into a biological outcome (cell cycle arrest, senescence or apoptosis). In cancer cells, the expression of MAGE correlates with reduced level of nuclear p53 and a strong impairment of p53 function and therefore MAGE proteins are thought to be involved into the impairment of p53 dependent response. (Adapted from Feng et al. Protein cell, 2011, Ladelfa et al. Cancer letters, 2012).

IV.3 MAGE-C2/CT10 a valuable vaccine target for MM

In MM, MAGE-C2 is frequently expressed in MGUS, solitary plasmocytoma, in MM bone marrow, MM osteolytic lesions and HMCL [141, 191, 250, 251, 277, 278]. Given its

expression pattern and antigenic property evidenced in melanoma and leukaemia patients by the spontaneous induction of anti-MAGE-C2 antibodies as well as its recognition by CTL clones [143, 263, 279-281], MAGE-C2 represents a potential marker for the diagnosis and prognosis as well as promising target for cancer immunotherapy. Therefore, a thorough understanding of the biological role of MAGE-C2/CT10 in the malignant phenotype of myeloma cells is worth pursuing.

CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS AND AIMS

Despite significant improvements in the treatment of multiple myeloma (MM), the median survival has not improved beyond 4-5 years and relapse will eventually occur in over 90% of patients initially responding to treatments. There is ample evidence that the drug resistance is due to the persistence of a small population of malignant plasma cells displaying stem cell properties including chemoresistance and clonogenic growth, which promote tumor regrowth. Therefore, the selective targeting of intracellular mechanisms crucially involved in promoting the survival of these resilient cells may lead to the eradication of both Myeloma precursors as well as their more differentiated progenitors.

Cancer-testis antigens belonging to the MAGE class I family of genes are commonly expressed in multiple myeloma (MM). The Expression of MAGE class I genes is often associated with an aggressive clinical course of the disease and resistance to chemotherapy, suggesting that MAGE genes may confer a survival advantage on myeloma cells. MAGE-C2, a member of the MAGE class I family of genes, is thought to be a good candidate for cancer immunotherapy given its very frequent expression in primary myeloma and immunogenicity. In cancer cells, MAGE-C2 may promote tumor growth by enhancing DNA repair and interfering with the function of the tumor suppressor p53, which plays a pivotal role in activating the programmed cell death in response to oncogenic stimulation and DNA damaging agents. These findings highlight the relevance of MAGE-C2-mediated p53 inactivation in tumors that do not frequently mutate p53 such as MM where p53 mutations or deletions are relatively rare and occur late in the pathogenesis. However, in spite of its frequent expression in MM, the biological role of MAGE-C2 in malignant plasma cells has never been elucidated and remains unclear.

We, therefore, investigated, for the first time, *in vitro*, the role of MAGE-C2 in tumor cells derived from patients with MM, by applying a transient and stable loss of function approach.

The effects of MAGE-C2 silencing on the survival of MM were examined by determining the number of viable cells, measuring proliferation by applying BrdU incorporation assay, analysing the anchorage independent growth ability of Myeloma cells, investigating the cell cycle phase distribution and apoptotic cell death activation by flow cytometry.

Since the expression of MAGE-C family is frequent in advanced stages of the disease, often associated with chromosomal deletions 17p13 with TP53 being the key gene in this site [277],

we addressed the question whether MAGE-C2 silencing may have an effect on the viability of myeloma cell lines expressing non-functional p53 or whether the hypothetical anti-apoptotic function of MAGE-C2 is only restricted to MM cells expressing wild-type (wt) p53. For this purpose, the mutational status of TP53 in the selected myeloma cell lines was determined by genomic-based sequencing.

Because a deep understanding of the exact mechanisms underlying the effects of MAGE-C2 depletion is prerequisite for the development of efficient MAGE-C2 targeting therapies, we characterized the molecular basis of the anti-proliferative and/or pro-apoptotic effects of MAGE-C2 silencing in myeloma cells bearing functional and non-functional p53 by applying pathway-focused gene and protein expression analyses of key molecules involved in the positive and negative regulation of cell cycle progression, DNA damage, repair and apoptosis.

Our combined findings regarding the biological function of MAGE-C2 would help to determine if this CTA represents a valuable therapeutic target for MM and how such target needs to be designed in order to achieve a clinical benefit for patients suffering from this fatal malignancy.

CHAPTER 3: MATERIALS AND METHODS

I. MATERIALS

I.1 Cell lines

Myeloma cell lines MOLP-8, KMS-12-BM, IM-9, RPMI-8226, NCI-H929, OPM-2, EJM, LP-1, U-266, SK-007 and AMO-I were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Human embryonic kidney 293T cells (HEK293-T) was obtained from Thermo Scientific Open Biosystem.

I.2 Cell culture conditions

MOLP-8, KMS-12-BM cell lines were cultured in RPMI 1640 Medium, GlutaMAX™ (Life technologies) supplemented with 20% Heat inactivated foetal bovine serum (FBS) (Life technologies) and 1% penicillin-Streptomycin (Pen-Strep) (Life technologies) . IM9, RPMI-8226, NCI-H929, OPM-2, U-266, SK-007 and AMO-I were cultured in RPMI 1640 supplemented with 10% FBS and 1% Pen-Strep. EJM and LP-1 were cultured in IMDM-GutaMAX™ supplemented with 10% FBS and 1% Pen-Strep. HEK293-T was maintained in DMEM, High Glucose, GlutaMAX™ (Life technologies) supplemented with 10% FBS, 1mM Sodium Pyruvate (life technologies), 20mM HEPES (Life technologies) and 1% Pen-Strep.

II. METHODS

II.1 DNA and RNA extraction

DNA and RNA were extracted from cells by using the GenElution Mammalian Genomic DNA miniprepKit (Sigma Aldrich, Germany) and the RNeasy Mini Kit (Qiagen, Germany), respectively, following the manufacturer's protocols. The concentration and the purity of RNA and genomic DNA were assessed by spectrometric measurement (Biophotometer, eppendorf).

II.2 Qualitative reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA was synthesized from 2 ug of denatured RNA (65°C for 5 min) by incubation at 37°C for 60 min with 3 ug oligo(dT), 1.5 units of AMV reverse transcriptase (Promega), 40 units of Rnasin (Promega), 10 mM from each dNTP (Invitrogene), 25 mM Mgcl2 (Promega) and 2 ul

AMV Reverse transcriptase 10X reaction buffer (Promega). Primer sequences used for the amplification of MAGE-C-2, p53 open reading frame and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, annealing temperatures and amplicon sizes are described in Table 4. All PCRs were carried out in 50 μ l PCR mixture containing 200 nM from each primer, 2.5 units of AmpliTaq Gold (Applied biosystems), 4 mM MgCl₂, 200 nM of dNTP and 200 ng of cDNA. The cycling conditions used for MAGE-C2, p53 and GAPDH amplifications were as follow: an incubation at 95°C for 10 min, 35 cycles of a denaturation at 95°C for 1 min, annealing at the appropriate annealing temperature for 1 minute (Table 4), an extension at 72°C for 1 min, with a final extension step of 72°C for 10 min. The amplification of MAGE-C2 full length cDNA was performed by “Touchdown” PCR as follows : 1 cycle of denaturation (10 min at 95°C), 14 cycles of denaturation (95°C for 1 min), annealing temperature (61°C for 1 min, with -0.5°C/cycle), and extension (72°C for 2min), followed by 35 cycles of denaturation (95°C for 1 min), annealing (54°C, 1 min), and extension (72°C for 2 min), and a final extension cycle of 72°C for 10 min. All RT-PCR experiments were performed at least twice and included controls without cDNA and cDNA synthesized from human testis RNA (Ambion, Austin, TX) using Mastercycler gradient (Eppendorf, Germany). Finally all amplicons were separated with 100 bp DNA marker (Life technologies) on 1.5% agarose gels, stained with ethidium bromide, visualized with UV light, recorded using a peqlab camera (Biotechnology GmbH) and transcript specificity was confirmed by sequencing of the PCR products in both directions.

II.3 Genomic based-sequencing of TP53 in HMCL

The mutational status of TP53 in the selected myeloma cell lines MOLP-8, OPM-2, SK-007, IM-9, U-266, RPMI-8226 and EJM was determined by genomic-based sequencing of fourteen amplicons across the entire TP53 coding region, the untranslated exon 1 and the A promoter. For this purpose, 14 primers, located at 20 bp from the ends of each exon was designed (Table 4) and used to amplify the genomic DNA by “Touchdown” PCR. PCR reactions were performed in 25 μ l PCR reaction mixtures containing, 2.5 U AmpliTaq Gold (Applied Biosystem), 200 nM primers, 200 nM of each dNTP and 4 mM MgCl₂. “Touchdown” PCR program used to amplify the exons was as follow: 1 cycle of denaturation (10 min at 95°C), 10 cycles of denaturation (95°C for 1 min), annealing temperature (T_m for 1 min, with -0.5°C per cycle), and extension (72°C for 1min), followed by 30 cycles of denaturation (95°C for 1 min), annealing (T_m, 1 min), and extension (72°C for 1 min), and a final extension cycle of 72°C for 10 min. Following 40 PCR cycles, amplicons were separated on 1.5% agarose gels,

stained with ethidium bromide, visualized with UV light, recorded using recorded using a peqlab camera and then extracted with Nucleospin Gel and PCR clean up Kit (Machery-Nagel). Next, the amplicons obtained by “touchdown” PCR were sequenced in either direction by Dye-terminator cycle-sequencing. A master mix of the following components was prepared in a total volume of 20 µl: Big Dye which contains Taq DNA polymerase, standard dNTPS, labelled ddNTPs, 10 pmol of a single primer, 3 to 10 ng of the purified PCR product and water. After an initial denaturation at 95°C for 10 minutes, the cycle sequencing reaction was run for 40 times with a denaturation at 96 C° for 10 seconds, annealing at 57 C° for 10 seconds and extension at 60 C° for 4 minutes. The labelled sequencing products was purified by ethanol precipitation and sequenced on the Applied Biosystem 3100 Genetic Analyzer in the Core Facility for Nucleic Acid Analysis at the Medical university of Hamburg. Finally, the sequences were analysed by using FinchTV viewer software and basic local alignment search Tool (BLAST).

Table 4. Primer sequences, annealing temperatures and product size for PCR amplification.

Gene	Primer sequence (5'→3')		Annealing temperature (°C)	Amplicon size (bp)
MAGE-C2/CT10	Outer For	AGGCGCGAATCAAGTTA	51	1359
	Outer Rev	TGGCATCATCTGCGGTATCA		
MAGE-C2/CT10	Inner For	GTGCTCCAGGAACCAGGT	54	1223
	Outer Rev	TGGCATCATCTGCGGTATCA		
MAGE-C2/CT10 LC	For	GGAGGGAGCACTTCGTCTATG	55	213
	Rev	AGGATGGAAAGGAAGACTAGGAACAG		
MAGE-C2/CT10 ORF <i>AgeI</i> RES <i>P2A</i>	For	<u>ACCGGT</u> ATGCCTCCCGTTCCAGGCGTTCATTC	62	1197
	Rev	<u>ACCGGTGGGGCCGGGGTTCTCTCCACGTCGCCGGCCTGC</u> <i>TTCAGCAGGCTGAAGTTGGTGGCGCCGCTGCC</i> TCAGAAAAGGAGACGTT		
P53ORF	For	AGTCTAGAGCCACCGTCCA	57	1450
	Rev	TTTCTTACTCCTTGGAGGCC		
TP53PA	For	CAGAGTGATAAGGGTTGTGAAGG	55	398
	Rev	CTGGCACAAAGCTGGACAGT		
TP53PB/E1	For	GGCGGATTACTTGCCCTTAC	55	291
	Rev	CGAGAGCCCCTGACTCAG		
TP53E2	For	CTGGATCCCCACTTTTCCTC	55	201
	Rev	CCCACAGGTCTCTGCTAGG		
TP53E3	For	AGCGAAAATTCCATGGGACTGAC	55	154
	Rev	CAGTCAGAGGACCAGTCTCAG		
TP53E4	For	CTGAGGACCTGGTCTCTGAC	55	364

	Rev	CCAGGCATTGAAGTCTCATGGA		
TP53E5	For	TTTCAACTCTGTCTCCTCCTCTT	55	248
	Rev	AGCCCTGTCGTCTCTCCAG		
TP53E6	For	CAGGCCTCTGATTCTCACT	55	185
	Rev	CTTAACCCCTCCTCCAGAG		
TP53E7	For	TCATCTGGGCCTGTGTTATC	55	166
	Rev	AGTGTGCAGGGTGGCAAG		
TP53E8	For	GCCTCTTGCTTCTCTTTCC	55	217
	Rev	TAACTGCACCCTTGGTCTCC		
TP53E9	For	GGAGACCAAGGGTGCAGTTA	55	204
	Rev	GAAAACGGCATTTTGAGTGTT		
TP53E10	For	ACTTCTCCCCCTCCTCTGTT	55	219
	Rev	GAAGGCAGGATGAGAATGGA		
TP53E11	For	TGTCATCTCTCCTCCCTGCT	55	205
	Rev	CAAGGGTTCAAAGACCCAAA		
TP53exE	For	AACTTCAGAACACCAACTTATACCA	55	203
	Rev	AGGTGTATTAATCCATTTTCAACTT		

II.4 Stealth™ RNAi siRNA transfection

For the transient loss of function, we used 3 distinct Stealth™ RNAi siRNA duplexes (Invitrogen) that target different sequences across MAGE-C2 coding region (Table 5) and two scrambled control Stealth™ RNAi siRNA. The day of transfection, myeloma cells were plated in 6 well plate (Cellstar, Greiner bio-one) at a density of 1.5 10E5 in 800 ul Optimem I reduced serum medium (Life technologies). 100 pmol of stealth™ RNAi siRNA duplex was diluted in 180 ul Optimem I and 3 ul of Lipofectamine™ 2000 (invitrogen) equilibrated at room temperature and mixed with 12ul Optimem. After 5 minutes incubation, diluted Stealth™ RNAi siRNA and diluted Lipofectamine™ 200 were mixed and incubated for another 20 minutes at room temperature. Afterwards, Stealth™ RNAi siRNA-lipofectamine™ 2000 complexes were added to each well dropwise to give a final Stealth™ RNAi siRNA concentration of 100 nM. The plate was mixed gently back and forth and incubated for 24h at 37°C in a CO2 incubator. The following day, 1 ml of a selective complete Medium was added to each well and incubated for another 24 or 48h for target validation.

II.5 Lentiviral vector production

For the stable loss of function, we used three lentiviral micro-RNA based vectors (pGIPZ, Thermo Scientific Open Biosystem, US) expressing constitutively under the pol II CMV-based promoter, a bi-cistronic transcript encoding for the green fluorescent protein (GFP) and short hairpin RNA (shRNA) cloned within microRNA30 (mir) backbone (shRNAmir) allowing the visual marking of cells expressing shRNAmir, which target three distinct

sequences on MAGE-C2 3' untranslated region (3'UTR) (Table 5). As a control, a construct targeting the housekeeping gene GAPDH (RHS4348, Thermo Scientific Open Biosystem) and a construct expressing scrambled sequence non targeting any known mammalian sequence (RHS4372, Thermo Scientific Open Biosystem) were used as a positive and negative control, respectively. Suspecting an anti-apoptotic function for MAGE-C2, we also performed a conditional RNAi-mediated gene silencing by using two IPTG-responsive lac repressor-operator mediated inducible shRNA lentiviral vectors (pLKO-puro-IPTG-3xLacO, Sigma-Aldrich). Under the control of a modified pol III human U6-based promoter, two shRNAs were targeting MAGE-C2 mRNA (Table 5) and one was a non-targeting shRNA control vector (SHC202, Sigma-Aldrich) as a negative control. For plasmid preparation, all clones were grown in LB-Lennox low salt media supplemented with 100µg/ml carbenicillin (Sigma-Aldrich) at 37°C for 18 hours with vigorous shaking. The plasmid DNA was extracted with maxi-prep kit following the manufacturer instruction (Invitrogen). Replication-deficient recombinant lentiviral particles were produced by transient transfection in HEK-293T cells using the calcium-phosphate method. Briefly, one day before transfection HEK-293T cells were seeded in 100 mm cell culture plate at a density of 5.5×10^6 per dish. In the following day 20 µg of each transfer vector was mixed with two additional plasmids required for the packaging: 10 µg of Gag/Pol-plasmid (psPAX2) and 2 µg of the envelope-plasmid, the vesicular stomatitis virus glycoprotein (pCMV-VSVG). The plasmid mix was diluted in water to 437.5µl and 50µl of CaCl₂ (2.5M) solution. The DNA/CaCl₂ mix was added to a precipitation buffer (2x HBS, PH=7.15) drop wise, while blowing air through the HBS with Pasteur pipette and the mixture was incubated at room temperature for 20 minutes. After having replaced the old medium with 10 ml new medium containing 25µM chloroquine (Sigma-Aldrich), the DNA mixture was added drop wise and cells were incubated overnight at 37°C. Following 14 to 16 hours, medium was changed with serum free media and cells were incubated overnight. After 24 hours, supernatants containing replication deficient viral particles were collected, filtered through 0.45µm syringe filter (VWR international) and stored in -80°C. To determine the biological titer of the lentiviral particles, producer cells were infected with serial of 5-fold dilutions. At 48 hours post-infection, the transduction efficiency of GFP-shRNAmir vector was assessed by flow cytometry based on the fluorescence of the reporter gene. The biological titer was calculated according to the following formula: TU (transducing units)/ml = $P \times N / V$, where P = %GFP+ cells, N = number of cells at time of transduction (5×10^4) and V = volume of dilution added to each well. pLKO-puro-IPTG-3xLacO viral particles not expressing any reporter gene were used to transduce

HEK-293T cells at different dilution and transduced cells were selected with puromycin (1ug/ml) (Sigma-Aldrich) at 48h post-transduction. After 10 days of selection, stably transduced cells were stained with 1% crystal violet solution (Sigma-Aldrich) and counted for the number of stained colonies.

Table 5. shRNA and siRNA sequences and sequence targets

Description	Sequence (5'→3') Color codes:mir-30 context sense loop antisense	Sequence target
GFP- MAGE-C2shRNAmir30#1 Clone :V3LHS_404218 (pGIPZ, Thermo Scientific OpenBiosystem)	TGCTGTTGACAGTGAGCG CTAGCTTCAGAGTGTTAATTTATAGTGAAG CCACAGATGTATAAATTAACACTCTGAAGC TAATGCCTACTGCCTGGA	1651-1671
GFP-MAGE-C2shRNAmir30#2 Clone: V2LHS-115108 (pGIPZ, Thermo Scientific OpenBiosystem)	TGCTGTTGACAGTGAGCG CGGAAATACCTTCTCCCTTATTAGTGAAG CCACAGATGTAAATAAGGGAGAAGGTATT TCCATGCCTACTGCTCGGA	1744-1764
GFP-MAGE-C2shRNAmir30#3 Clone: V2LHS-115109 (pGIPZ, Thermo Scientific OpenBiosystem)	TGCTGTTGACAGTGAGCG CAGTTAAATAGTGGAACAATAGTGAAGCC ACAGATGTATTGTTCCACTATTTAACTGTG CCTACTGCTCGGA	1839-1857
RFP-MAGE-C2shRNAmir30 Clone:V2THS_115108 (pTRIZ, Thermo Scientific OpenBiosystem)	TGCTGTTGACAGTGAGCG GAAATACCTTCTCCCTTATTAGTGAAGCCA CAGATGTAAATAAGGGAGAAGGTATTTCTG CCTACTGCTCGGA	1745-1763
pLKO-puro-IPTG-3xLacO shRNA#92 Clone:TRCN0000115792 (Sigma-Adrich)	GCAGTTAAATAGTGGAACAAA TTTGTTCCTACTATTTAACTGC	1838-1853
pLKO-puro-IPTG-3xLacO shRNA#93 Clone:TRCN0000115793 (Sigma-Adrich)	CGCCTCTTCCACTTTGTACTT AAGTACAAAGTGGAAGAGGCG	471-491
MAGE-C2HSS122200 (Stealth™ RNAi siRNA#1) (Invitrogene)	GAUACCGCAGAUGAUGCCAGUGUCA UGACAGUGGCAUCAUCUGCGGUAUC	1393-1417
MAGE-C2HSS122201 (Stealth™ RNAi siRNA#2) (Invitrogene)	CCGACCUCAGUUGAGUUAGAAGACU AGUCUUCUAACUCAACUGAGGUCGG	397-421
MAGE-C2HSS122202 (Stealth™ RNAi siRNA#3) (Invitrogene)	GCCCUCUGGUGUGAUACCAAUCUU AAGAUUUGGUAUCACACCAGAGGGC	561-585

II.6 Transduction of myeloma cell lines

To determine the multiplicity of infection (MOI) required for optimal expression of GFP-shMAGE-C2mir and pLKO-puro-IPTG-3xLacO shRNA for each myeloma cell line, cells were harvested and resuspended in media supplemented with 8 ug/ml polybrene (hexadimethrine bromide; Sigma-Aldrich) and lentiviral particles were added at different MOI (5, 10, 15, 20). 48h post-transduction, GFP-shMAGE-C2mir transduced myeloma cells were assessed for the transduction efficiency by flow cytometry for GFP expression. For target validation, myeloma cells transduced with GFP-shMAGE-C2mir or pLKO-puro-IPTG-3xLacO shMAGE-C2 constructs, at each MOI, were analysed by quantitative RT-PCR and Western blot, at 48h and 72h post-transduction.

II.7 SYBER Green based quantitative RT-PCR

For validation of MAGE-C2 mRNA cleavage, total RNA from cells collected at 48 or 72h post-transduction or post-transfection was extracted using the RNeasy kit (Qiagen) and transcribed into cDNA as described above. For the mRNA quantification, a master mix of the following components was prepared at the final concentrations indicated: 4.0 mM MgCl₂, 400 nM forward and reverse primers (MAGE-C2 LC, Table 4), 200 nM dNTPs (Invitrogen), 1% dimethylformamide, 2.5 mg/ml BSA, SYBR Green I (Sigma, St. Louis, MO) diluted 1:1000, and 1 Unit FastStart Taq polymerase (Roche Diagnostics, Branchburg, NJ) in a total volume of 20 µl and run in Lightcycler (Roche). After an initial denaturation at 95°C for 10 minutes, PCR reactions were run 40 times with a denaturation at 95 C° for 1 min, annealing at 55°C for 1 minute and extension at 72 C° for 1 minute. Fluorescence intensity was measured at the end of each elongation phase. In order to determine specificity of the PCR reaction, a melting curve analysis was carried out immediately after amplification. The relative expression of MAGE-C2 mRNA was calculated by 2- $\Delta\Delta$ Ct method using GAPDH mRNA expression level for normalization. All experiments were performed in duplicate three times.

II.8 Western blot analysis

Whole cell proteins were extracted from untreated, transfected or transduced cell lines using cell lysis buffer (Bio vision) supplemented with a cocktail of protease Inhibitors (Roche). Proteins concentrations were measured by Bradford assay using Quick Start™ Bradford 1X Dye Reagent (Bio Rad) and a spectrophotometer (GeneQuant pro) set at 595 nm and determined using a BSA standard curve. For the separation of small to medium sized proteins,

10ug of denaturated samples and a prestained protein Marker (peqGold Protein-Marker V) were run on Nupage® 4-12% Bis-tris polyacrylamide gels (life technologies) in MES SDS running buffer (Life technologies). For large molecular weight protein, 10ug of denaturated cell lysates were run with the HiMark™ pre-stained high molecular weight standard (Catalog no. LC5699, Invitrogen, life technologies) on Nupage®3-8% Tris-Acetate gels in Tris-Acetate SDS Running Buffer. Electrophoresed samples were transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked for an hour with 5% non-fat milk blocking solution and incubated overnight at 4°C with MAGE-C2 mouse monoclonal antibody CT10 (Clone CT10#5) [282] (kindly provided by the New York branch of the Ludwig Institute for Cancer Research), β Actin mouse monoclonal antibody (C4): sc-47778 (Santa Cruz) or other appropriate anti-body diluted (1:1000) in 5% BSA-TBS-T buffer. The following day, membranes were incubated for 1 hour with a horseradish peroxidase-conjugated anti-Mouse (#7076, Cell signaling) or anti-Rabbit (#7074, Cell signaling) IgG diluted (1:2000) in 5% non-fat milk solution. Finally, protein bands were detected with an enhanced chemiluminescence Western blot substrate (ECL, Amersham).

II.9 BrdU incorporation assay

To analyze the effect of MAGE-C2 silencing on myeloma proliferation, we assessed DNA synthesis by applying a colorimetric assay based on the incorporation of the thymidine analogue, 5-bromo-2'deoxyuridine (BrdU), into the DNA of proliferating cells. Therefore, 72h post-transduction, cells were counted and plated into 96-well tissue culture plate (Cellstar, Greiner bio-one) at the following density: 10E4 (MOLP-8), 2x10E4 (U-266, SK-007, OPM-2), 5x10E4 (IM-9 and LP-1), 8X10⁴ (EJM, RPMI). The following day, cells were labeled with BrdU, incubated for 4 hours and the quantification of cell proliferation was determined by an enzyme-linked immunoabsorbant assay, (the Biotrack™ cell proliferation ELISA system, Amersham) and an ELISA reader at 450nm. All the procedure followed the manufacturer's protocols and each experiment was performed in triplicate and repeated three times.

II.10 Viability assay

The growth and viability of myeloma cell lines transduced with targeting and non-targeting constructs were daily monitored by inverted phase/fluorescence microscopy (Carl Zeiss) and by counting cells that excluded trypan blue (Sigma, Germany) over 4 to 6 days from the day of transduction. In some selected experiments, cell viability was determined by MTT assay (ATCC). At 96h post transduction, cells were plated into 96-well tissue culture plate (Cellstar,

Greiner bio-one) at the following density: 10E4 (MOLP-8), 2x10E4 (U-266, SK-007, and OPM-2), 5x10E4 (IM-9 and LP-1), 8X104 (EJM, RPMI). The following day, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was added to cells and incubated for 2 to 4 hours at 37°C and periodically viewed under an inverted phase microscope (Carl Zeiss). The reduced MTT corresponding to the intracellular purple formazan, produced by metabolically active cells was then solubilized with a detergent and quantified by an ELISA reader (Sunrise- TECAN) at 570nm.

II.11 Cell cycle and apoptosis assays

To determine the effect of MAGE-C2 silencing on cell-cycle progression, a BrdU/7AAD bivariate analysis was performed using BrdU Flow Kit (BD Pharmingen™). Therefore myeloma cells were synchronized in low serum culture condition by infection with lentiviral particles in medium supplemented with 0.5% FCS for 13 hours (MOLP-8 and IM-9) or 24hours (U-266, SK00-7, EJM and RPMI-8226) and then induced with fresh medium supplemented with 20 or 10 % FBS and incubated for another 37h (MOLP-8 and IM-9) and 24h (U-266, SK007, EJM and RPMI-8226). At 48h post-transduction, myeloma cells were pulsed with BrdU every 24h (48h, 72h, 96h and 120h post-transduction) for 1 hour. BrdU uptake and total DNA content per cell were stained according to the manufacture's protocol. To analyze apoptosis, cells were collected at 120h post-transduction and stained with Annexin V-APC and 7-AAD (BD Pharmingen™) diluted in Annexin binding buffer. The cell cycle distribution and the percentage of apoptotic cells were assessed with FACSCalibur flow cytometer (BD Biosciences) and analyzed with flowjo. Each assay was repeated three times

II.12 MAGE-C2/CT10 Rescue assay

MAGE-C2 cDNA lacking the 3'Untranslated region, which is targeted by the shRNAmir lentivirus, was amplified and hence the exogenous MAGE-C2 transcript will not be targeted by shRNA and can rescue the knockdown phenotype. To avoid multiple vector infections and ensure a simultaneous expression of both the exogenous MAGE-C2 and shMAGE-C2mir, we sub-cloned MAGE-C2Δ3'UTR cDNA in a doxycycline regulated Tet-on lentiviral inducible RFP-shRNAmir vector (pTRIPz, Thermo Scientific Open Biosystem) (Table 5) at the single AgeI restriction site, upstream and in-frame of the bi-cistronic TurboRFP-shRNAmir transcript. To ensure a stoichiometric expression of MAGE-C2Δ3'UTR and TurboRF-shRNAmir, we created a tri-cistronic MAGE-C2Δ3'UTR-TurboRF-shRNAmir transcript by linking MAGE-C2Δ3'UTR C-terminus to the Turbo-RFP N-terminus with the porcine

Teschovirus_1 virus 2A (P2A) ribosomal skipping peptide which mediates a co-translational cleavage and stoichiometric co-expression of both MAGE-C2 Δ 3'UTR and the downstream TurboRFP-shRNAmir. This procedure enabled us to track both shMAGE-C2mir and MAGE-C2 Δ 3'UTR expression following doxycycline induction through the visual marking of cells expressing the red fluorescent protein (RFP). The packaging of MAGE-C2 Δ 3'UTR-TurboRFP-shMAGE-C2mir, TurboRFP-shMAGE-C2mir and TurboRFP-Non-specific-shRNAmir (RHS4743, Thermo Scientific Open Biosystem) inducible vectors as well as the transduction of myeloma cells were performed following the same conditions described above. Because of the low viral titer of Tet-on lentiviral inducible RFP-shRNAmir vector, the viral supernatant of each construct was concentrated by ultracentrifugation in a swinging-bucket ultracentrifuge rotor (Sorvall HB-6, Thermo Scientific) at 7000g overnight at 4°C. The following day, the pellet was resuspended in small volume of DMEM medium and assessed for the biological titer by flow cytometry, as described above. Transduced myeloma cells were treated with puromycin (1 μ g/ml) for 10 days, to select for the cells having integrated stably MAGE-C2 Δ 3'UTR-TurboRF-shMAGE-C2mir, TurboRF-shMAGE-C2mir or control vectors, and then induced with an analogue to tetracycline, doxycycline (2 μ g/ml) (Sigma-Aldrich). After 4 days of induction, cells were collected and assessed for Western blot target validation.

II.13 Colony formation assay

To assess the anchorage-independent growth ability of myeloma cells following MAGE-C2 depletion, 72h post-transduction, myeloma cells were collected, counted, diluted in 0.3 ml complete medium at the following density: 1X10E4 (MOLP-8, U-266), 5X10E4 (SK-007, IM-9, EJM, RPMI-8226, OMP-2) and then mixed with 3 ml Methylcellulose-Based Media (Methyl Cult™ H4034 Optimum, stem cell technologies) for duplicate cultures (1:10 ratio). The mix was vortexed vigorously and let stand for at least 5 minutes to allow bubbles to dissipate. Afterwards, myeloma cell-Methylcellulose-Based Media mix were plated in 6 well plate tissue culture dishes (1.5 ml/well) and cultured at 37°C in humidified incubator with 5% CO₂ for 10 to 15 days after plating. Colonies, consisting of at least 40 cells, were counted under inverted phase/ fluorescence microscope (Carl Zeiss). All experiments were performed in duplicate and repeated three times.

II.14 Pathway-focused gene expression analysis by quantitative PCR arrays

We used two RT2 Profiler™ PCR array (SABioscience, Qiagen) that profiles the expression of 86 genes regulating cell cycle transition, checkpoint and arrest (Human Cell cycle array, PAHS, 020Z) and 84 genes regulating the apoptosis (Human apoptosis, PAHS, 012Z). IM-9 U-266 and EJM myeloma cells transduced with GFPshMAGE-C2mir or GFP-Non-specific-shRNAmir were harvested at 72hours post-infection for total RNA extraction using the RNeasy Mini Kit (Qiagen, Germany). Following the assessment of the concentration and quality of RNA by spectrometric measurement (Biophotometer, eppendorf), cDNA was synthesized with RT2 first strand kit (Catalogue no. 330401, Qiagen) using 500ng of total RNA from each sample for 96-well plate formats following the manufactures' instructions and used afterwards as a template for SYBR Green based real-time PCR using RT2 SYBR Green Master mixes (Catalog no. 330502, Qiagen) that contains HotStart DNA Taq Polymerase, PCR Buffer, dNTPS mix and SYBR Green dye. The 96 well RT2 profiler PCR array containing PCR components mix were placed in the real-time cycler (Bio-Rad, MJ Research Chromo4) and run, after an initial denaturation at 95°C (10 minutes), for 40 cycles as follow: a denaturation at 95 C° for 15 seconds, annealing at 55°C for 30 seconds and extension at 72 C° for 30 seconds. Fluorescence intensity was measured at the end of each elongation phase. In order to determine the specificity of the PCR reaction, a melting curve analysis was carried out immediately after amplification. The threshold cycle values were analyzed with the SABiosciences PCR Array Data analysis Web-based software that performs pair-wise comparison between groups (GFP-shMAGE-C2mir transduced cell versus GFP-Non-specific-shRNAmir transduced cells). The fold change for each pathway-focused gene was calculated by $2^{(-\Delta\Delta Ct)}$ method using the Ct values for the gene of interest and the appropriate housekeeping gene chosen for normalization: Fold change ($2^{(-\Delta\Delta Ct)}$) is the normalized gene expression ($2^{(-\Delta Ct)}$) in GFP-shMAGE-C2mir positive cells divided the normalized gene expression ($2^{(-\Delta Ct)}$) in GFP-NS-shRNAmir positive cells. Genes with average Ct values greater than the cut-off value (35) were considered as not detected. Genes with fold regulation greater than 1.5 were considered up-regulated in comparison to the control group, while genes with fold regulation less than 1 were considered down-regulated in comparison to the control group. All experiments were performed three times. The p values were calculated based on a Student's t-test. P values less than 0.05 were statistically significant.

II.15 Statistics

All data are presented as the mean values of three independent experiments \pm S.D. All graphs were performed with Graph Pad prism 5. To test for statistical significance, the paired t-test was applied and statistical significance was accepted with *P< 0.05, **P<0.01, ***P<0.001.

CHAPTER 4: RESULTS

I. MAGE-C2/CT10 is constitutively expressed in Myeloma cell lines

As prerequisite for the functional analysis of MAGE-C2 in myeloma, we first determined its expression in 11 myeloma cell lines and PBMC from 8 healthy donors using nested PCR (Figure 3A). We detected two MAGE-C2 splicing variants in 10 out of 11 myeloma cell lines and in normal testis tissue (Figure 3B), which served as a positive control, but not in PBMC from healthy donors (Figure 4A). Next, we confirmed by western blot the presence of MAGE-C2 protein, indicated by two bands with a molecular weight between 49kD and 55kD in myeloma cell lines and testis lysate expressing MAGE-C2 mRNA (Figure 3C). No reactivity was detected neither in MAGE-C2 RNA-negative myeloma cell line LP-1 nor in healthy PBMC (Figure 4B). Specificity of immunoblotting was confirmed by the ectopic expression of the MAGE-C2 open reading frame in HEK-293T cells followed by western blot analysis (Figure 3D). These findings indicate that MAGE-C2 is constitutively expressed in the vast majority of myeloma cell lines suggesting a potential role of this protein in the malignant phenotype of MM.

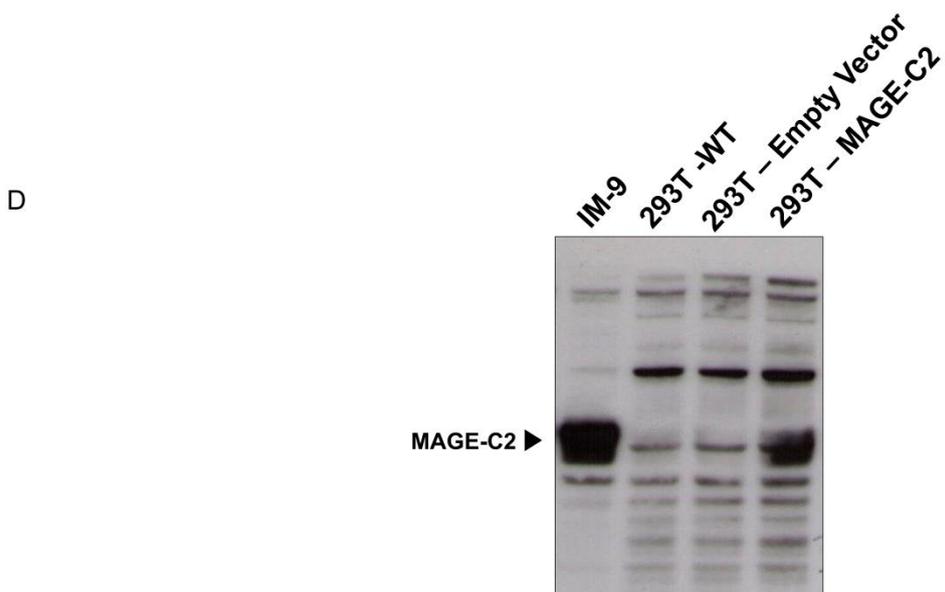
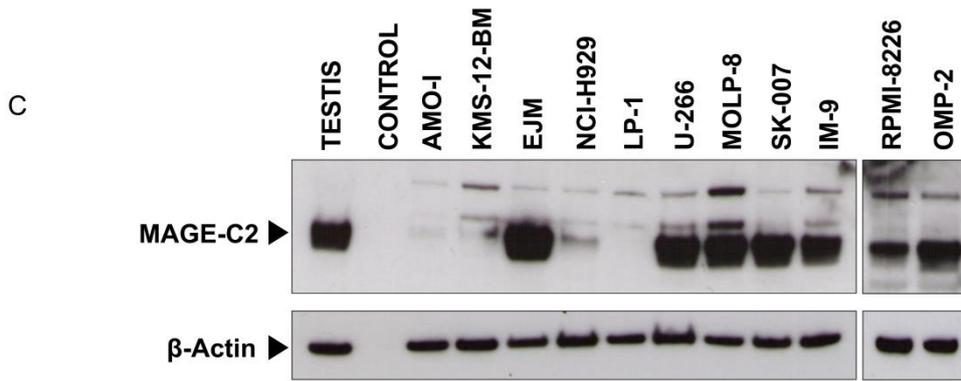
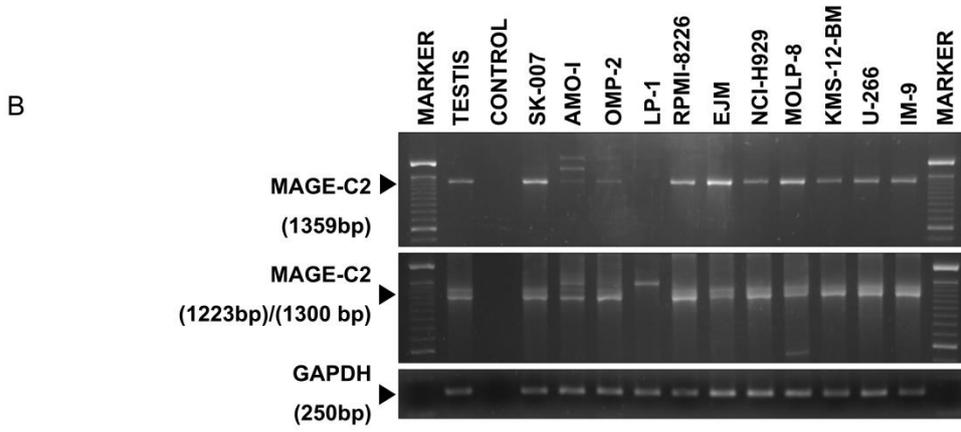
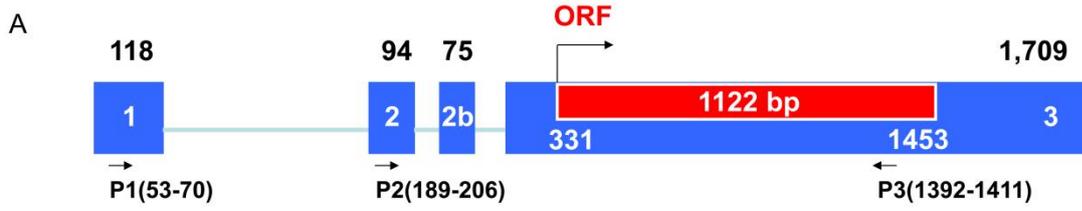


Figure 3. The analysis of MAGE-C2/CT10 expression in myeloma cell lines. (A) Schematic presentation of MAGE-C2 gene and the primers used for MAGE-C2 mRNA expression. (B) The analysis of MAGE-C2 mRNA expression in myeloma cell lines by Nested RT-PCR. (C) Western blot analysis of MAGE-C2 protein expression in Myeloma cell lines and adult healthy testis. (D) The analysis of the ectopic expression of MAGE-C2 in HEK-293T cell line by Western Blot. β -Actin served as a loading control. Testis served as positive controls. (P: primers)

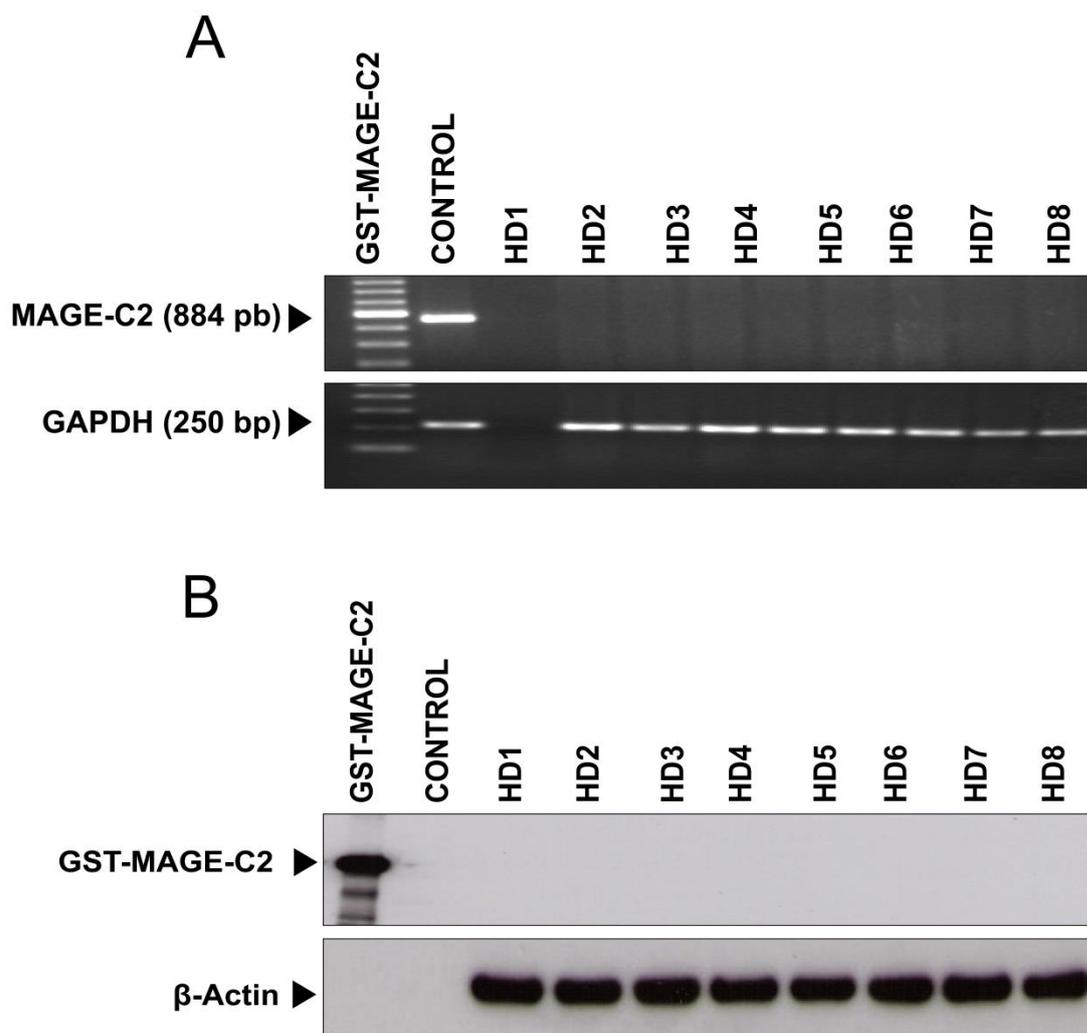


Figure 4. The analysis of MAGE-C2/CT10 expression in PBMC from healthy donors. (A) The analysis of MAGE-C2 mRNA expression by Nested RT-PCR in PBMC from Healthy donors. (B) Western blot analysis of MAGE-C2 protein expression in PBMC from healthy donors. β -Actin served as a loading control. Testis and GST-MAGE-C2 recombinant protein served as positive controls. (PBMC: peripheral blood mononuclear cells, HD: Healthy donors)

II. TP53 mutations do not preclude MAGE-C2/CT10 expression in MM

Knowing that MAGE-C2 is a negative regulator of p53 function, being frequently expressed at the advanced stage of MM during which p53 mutation and/or deletion are common [277], we can assume that the inactivation of p53 may not preclude the overexpression of MAGE-C2. Consequently, this latter would exert its presumed anti-apoptotic role in p53 independent fashion. To demonstrate this hypothesis, the mutational status of TP53 in some selected myeloma cell lines constitutively expressing MAGE-C2 was determined by genomic-based sequencing of the entire TP53 coding region, the Untranslated Exon 1 and the A promoter (Fig. 3A, B). The functional significance of the detected mutations was analysed based on findings published in the International Agency for Research on Cancer (IARC) TP53 mutation database and summarized in Table 6 and Figure 12.

We found that wild-type p53 (wt-p53) was only present in two myeloma cell lines (MOLP-8 and IM-9) (Figure 6A, B), while 5 cell lines expressed mutated p53. Only single biallelic missense mutations were detected in all p53 mutant myeloma cell lines and there were mainly clustered in the most conserved parts of Exon 5 (U-266, SK007, OPM-2, EJM) and exon 8 (RPMI-8226). Exons 5-8 encode for residues 130–286 which contains the most important region for the folding and the stabilization of p53 as well as the site-specific DNA-binding site in which most tumor-associated mutations are found. These mutations either prevent normal DNA contact (functional mutation) or alter the structural integrity and stability of the DNA binding interface or the β sandwich (conformational mutation) [283]. In both U-266 and SK-007 (Figure 7A, Figure 8A), the missense mutation in exon 5 is a transition of G to A at the first base of codon 161 substituting the Alanine (A) residue to Threonine (T). A161T mutation is located at the S4 strand at close proximity to the L2 loop and the position of residue 161 maintains the structural integrity of the β scaffold which is required for the folding and the stabilization of this DNA-binding domain [284]. Although A161T mutation may cause local deformation in structure, it does not have dramatic effect on the DNA binding interface of p53 which retains a partial transcriptional activity [285]. In OPM-2 cell line, the missense mutation is a transition of G to A at the second base of codon 175 substituting the Arginine (R) residue to Histidine (H) (Figure 9A). The R175H is a hot spot conformational mutation located at the L2/L3 loops that interact with the minor groove of DNA molecule which may alter wt-p53 conformation and lead to a total loss of the

transcriptional activity [286]. In EJM cell line, the missense mutation at exon 5 is a transversion of G to C at the third base of codon 132, substituting the lysine (K) residue to an Asparagine (N) (Figure 10A). K132N is a cold spot missense mutation, located in a conserved region S2' strand of the β scaffold, which may alter the structural integrity. According to the IARC p53 database, K132N is predicted to lead to a total loss of the transcriptional activity. In RPMI-8226, a transition of G to A at codon 285 substituting the Glutamic acid residue (E) to a lysine (K) (Figure 11A). The E285K missense mutation is localised at the conserved region H2 helix (272 -287) required for DNA contact [284]. Both missense mutations K132N (EJM) and E285K (RPMI-8226) have been reported to induce temperature sensitive conformational mutants that lose completely their transcriptional activity at non permissive condition (37°C) and above due to an instability of the correct protein folding at the body temperature and wt p53 activity is restored at permissive conditions (32 C°) [287, 288].

Additionally, we determined the status of codon 72 polymorphism, which is located on the proline rich domain, an essential domain for a full p53-dependent apoptotic response. Knowing that codon 72 encodes either the ancestral form Proline (CCC) or Arginine (CGC) [289], in our study, the arginine residue at codon 72 was detected in all cell lines bearing wild type or mutated p53 except for RPMI-8226 cell line that bears the proline residue on both p53 alleles. Furthermore, the expression of p53 was investigated in all myeloma cell lines by RT-PCR (Figure 5C) and the specificity of the amplified band was confirmed by sequencing. Using a polyclonal antibody, western blot analysis detected both wt and mut-p53 in all myeloma cell lines expressing constitutively MAGE-C2 (Figure 5D). These combined findings indicated to us that if MAGE-C2 may confer a biological advantage to the malignant phenotype of MM, it might not be strictly p53-dependent.

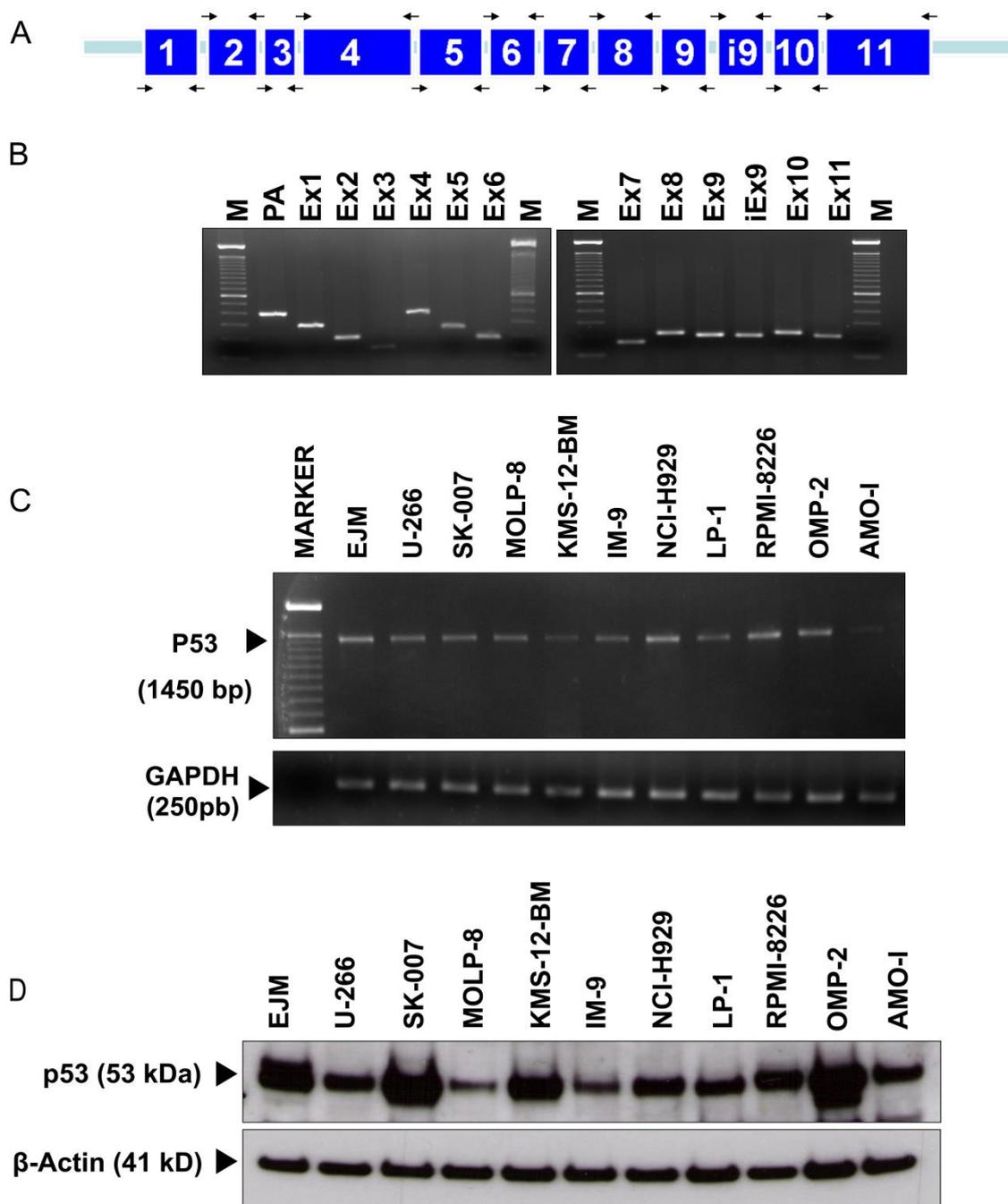


Figure 5. The analysis of TP53 mutational and expression status in myeloma cell lines. (A) Schematic presentation of TP53 gene and the primers used for the genomic-based sequencing. The mutational status of TP53 in myeloma cell lines MOLP-8, OPM-2, SK-007, IM-9, U-266, RPMI-8226 and EJM was determined by genomic-based sequencing of fourteen amplicons across the entire TP53 coding region, the untranslated exon 1 and the promoter A (PA) by designing primers that anneal to sequences located at 20 bp from the ends of each exon. (B) Ethidium bromide stained PCR products of all TP53 Exons, PA after gel electrophoresis and amplified using as a template genomic DNA extracted from MOLP-8 Myeloma cell line. (C) Qualitative RT-PCR analysis of p53 open reading frame expression in Myeloma cell lines. (D) Western blot analysis of p53 protein expression in Myeloma cell lines.

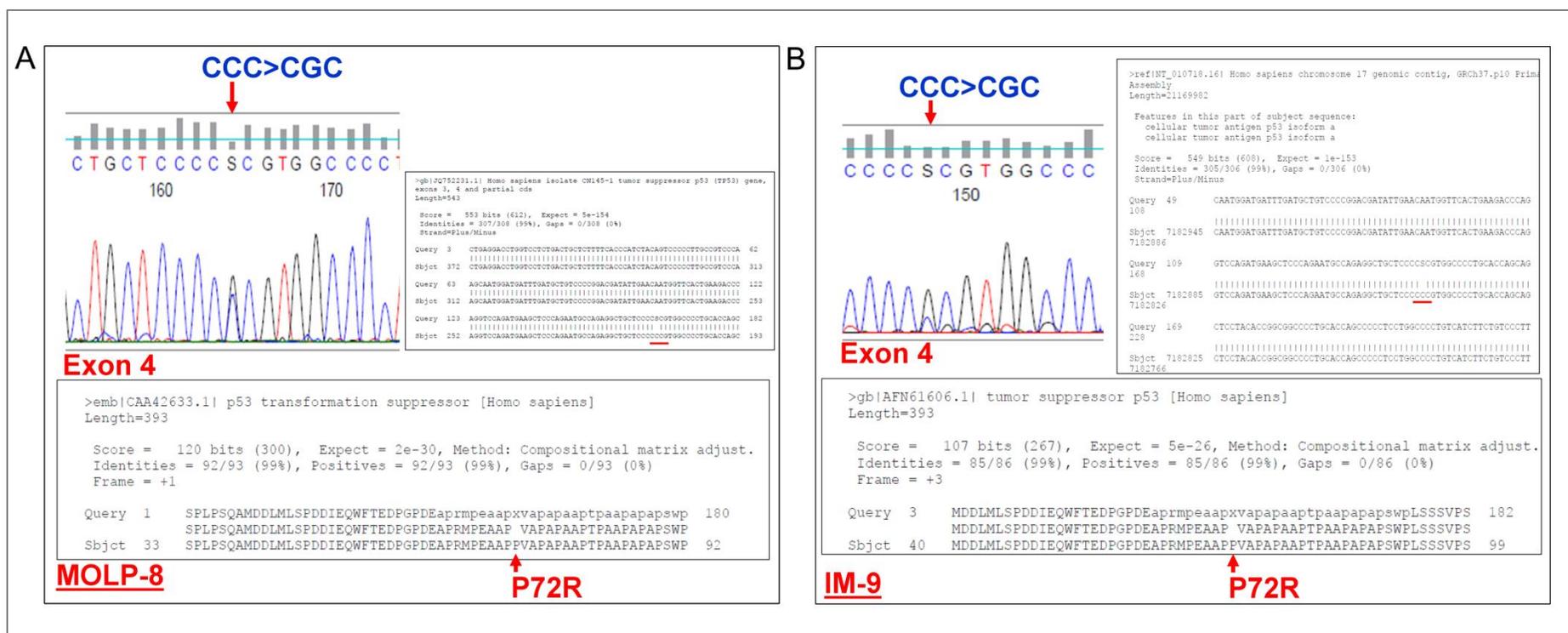


Figure 6. The mutational status of TP53 in MOLP-8 and IM-9 myeloma cell lines. Screen shot of the TP53 DNA Sequence chromatogram, the called bases and their alignments to contig assembly sequences as well as protein sequence alignments. **(A)** The sequence analysis of TP53 in MOLP-8 shows the presence of the arginine allele at codon 72 at Exon 4 (P72R) on both alleles. **(B)** The sequence analysis of TP53 in IM-9 shows the presence of the arginine codon on Exon 4 (P72R).

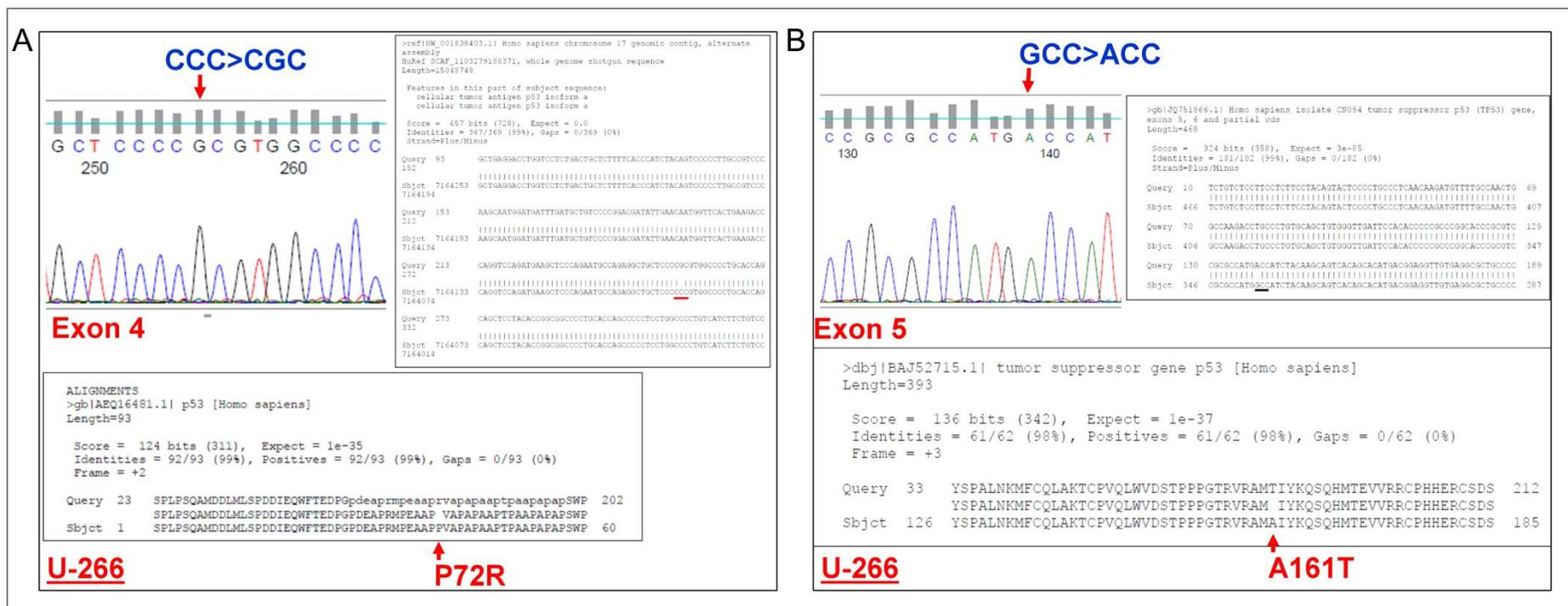


Figure 7. The mutational status of TP53 in U-266 myeloma cell line. Screen shot of the TP53 DNA Sequence chromatogram, the called bases and their alignments to contig assembly sequences as well as protein sequence alignments. **(A)** The sequence analysis of TP53 in U-266 shows the presence of the arginine allele at codon 72 on Exon 4 (P72R). **(B)** The sequence analysis of TP53 in U-266 shows the presence of the bi-allelic missense mutation A161T on exon 5.

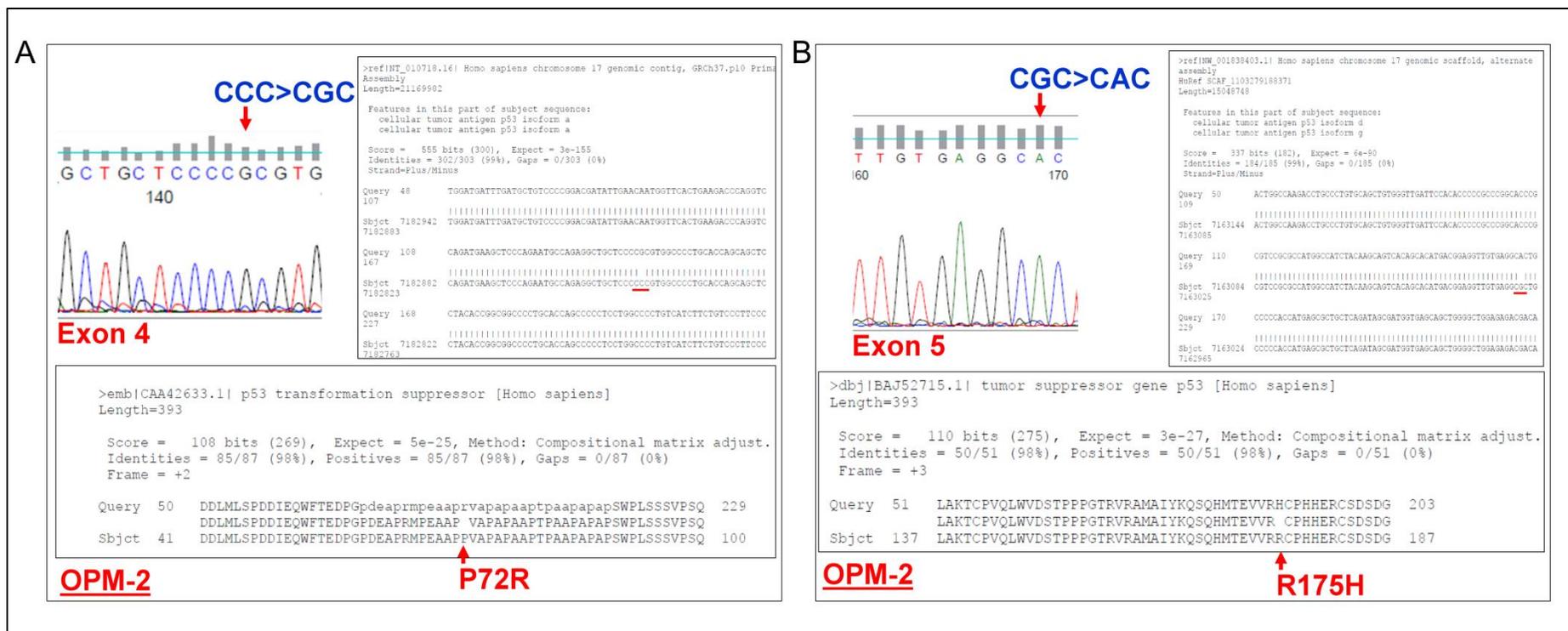


Figure 9. The mutational status of TP53 in OPM-2 myeloma cell line. Screen shot of the TP53 DNA Sequence chromatogram, the called bases and their alignment to contig assembly sequences as well as protein sequence alignment. **(A)** The sequence analysis of TP53 in OPM-2 shows the presence of the arginine allele at codon 72 on Exon 4 (P72R). **(B)** The sequence analysis of TP53 in OPM-2 shows the presence of the bi-allelic missense mutation R175H on exon 5.

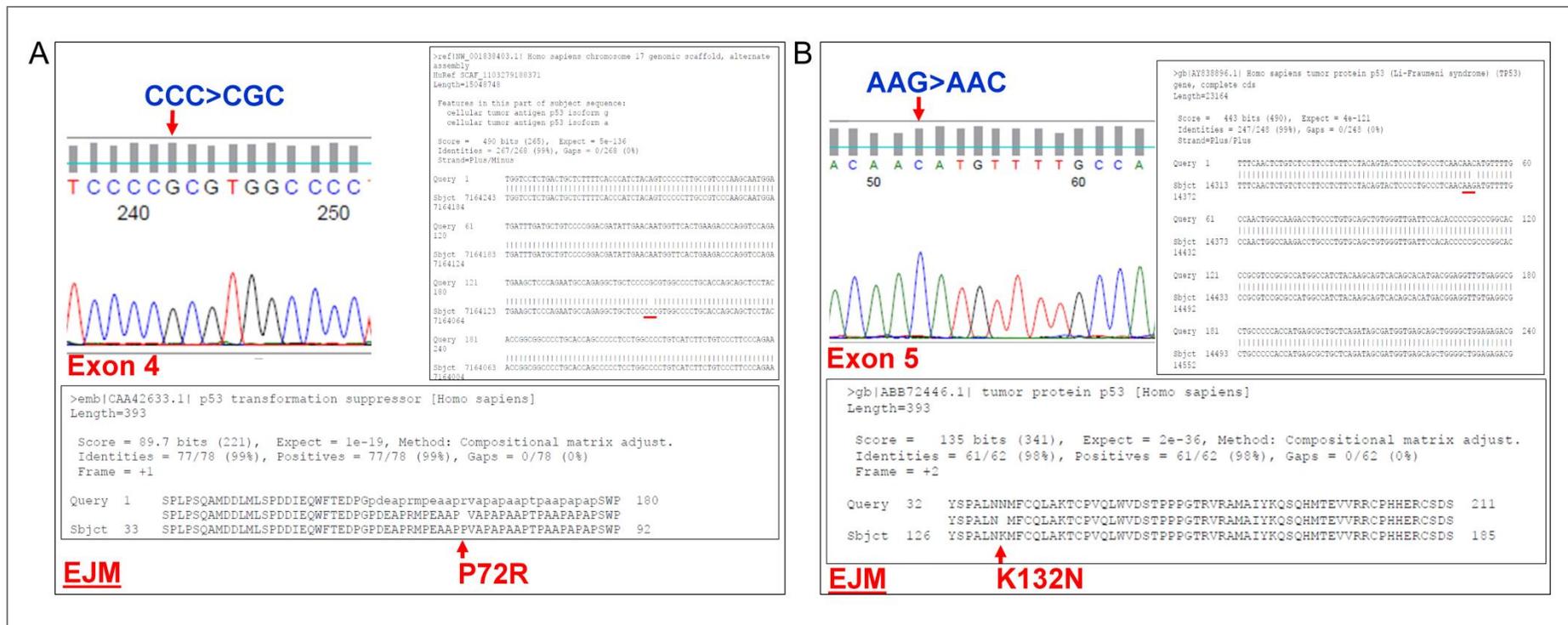


Figure 10. The mutational status of TP53 in EJMY myeloma cell line. Screen shot of the TP53 DNA Sequence chromatogram, the called bases and their alignments to contig assembly sequences as well as protein sequence alignments. **(A)** The sequence analysis of TP53 in EJMY shows the presence of the arginine allele at codon 72 on Exon 4 (P72R). **(B)** The sequence analysis of TP53 in EJMY shows the presence of the bi-allelic missense mutation K132N on exon 5.

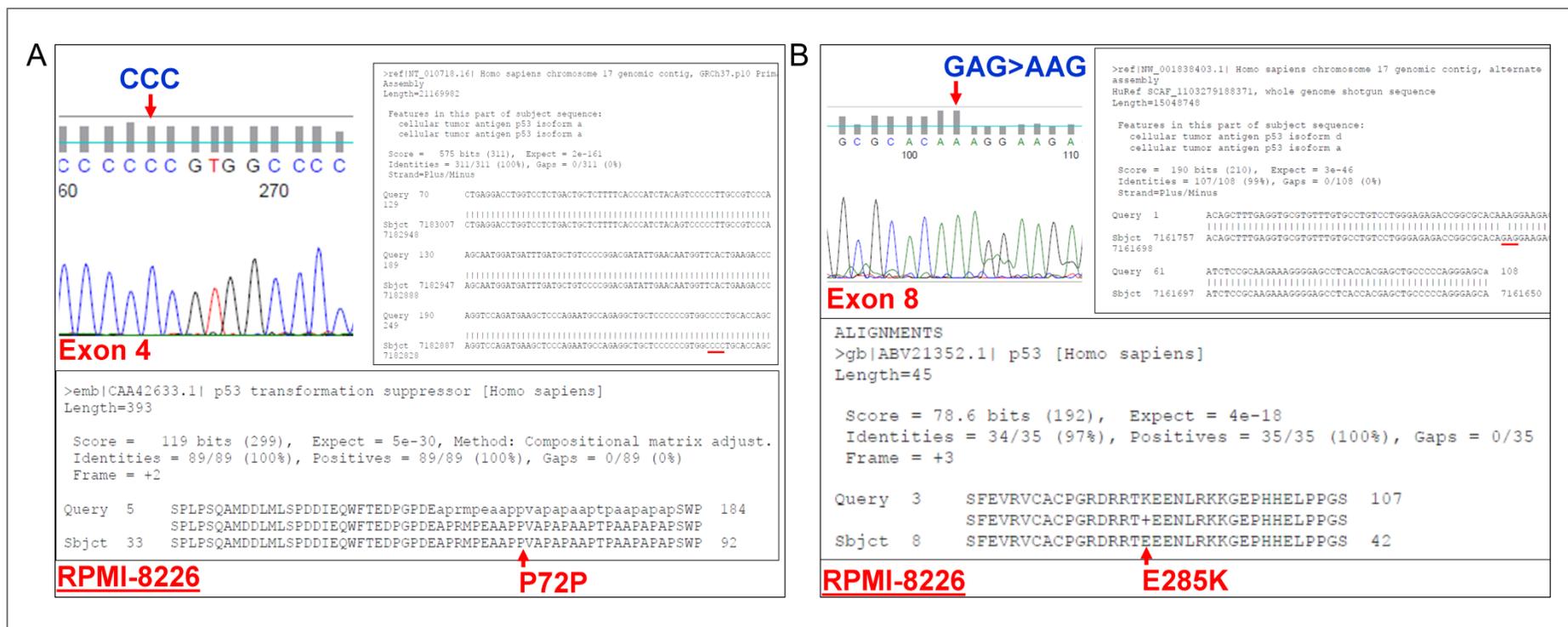


Figure 11. The mutational status of TP53 in RPMI-8226 myeloma cell line. Screen shot of the TP53 DNA Sequence chromatogram, the called bases and their alignments to contig assembly sequences as well as protein sequence alignments. **(A)** The sequence analysis of TP53 in RPMI-8226 shows the presence of the proline allele at codon 72 on Exon 4 (P72). **(B)** The sequence analysis of TP53 in RPMI-8226 shows the presence of the biallelic missense mutation E285K on Exon 8.

Table 6. TP53 mutational status in myeloma cell lines

Cell lines	Exons	Codon description (cDNA) (a)	Amino acid description (a)	Mutation type/ SNP	Domain Function and structural motif	Structural function (b)	Transactivation class (b)	P53 status	p53 protein expression
MOLP-8	4	c.215C>G	p.P72R (wt/mut)	Missense/SNP	Proline-Rich domain	Functional	Functional	Wild type	+
IM-9	4	c.215C>G	p.P72R (wt/mut)	Missense/SNP	Proline-Rich domain	Functional	Functional	Wild type	+
U-266	4	c.215C>G	p.P72R (mut/mut)	Missense/SNP	Proline-Rich domain	Non-functional	Partially functional	Conformational mutant	+
	5	c.481G>A	p.A161T (mut/mut)	Missense	DNA binding domain (NDBL/beta sheets)				
SK-007	4	c.215C>G	p.P72R (mut/mut)	Missense/SNP	Proline-Rich domain	Non-functional	Partially functional	Conformational mutant	+
	5	c.481G>A	p.A161T (mut/mut)	Missense	DNA binding domain (NDBL/beta sheets)				
OPM-2	4	c.215C>G	p.P72R (mut/mut)	Missense/SNP	Proline-Rich domain	Non-functional	Non functional	Conformational mutant	+
	5	c.524G>A	p.R175H (mut/mut)	Missense	DNA binding domain (L2/L3)				
EJM	4	c.215C>G	p.P72R (mut/mut)	Missense/SNP	Proline-Rich domain	Non-functional	Non functional	Conformational Mutant (Temperature sensitive)	+
	5	c.396G>C	p.K132N (mut/mut)	Missense	DNA binding domain (L1/S/H2)				
RPMI-8226	4	c.215C	p.P72P (mut/mut)	Missense/ SNP	Proline-Rich domain	Non-functional	Non functional	Conformational Mutant (Temperature sensitive)	+
	8	c.853G>A	p.E285K (mut/mut)	Missense	DNA binding domain (L1/S/H2)				

(a) Annotations available in the IARC TP53 DATA BASE

(b) Transactivation activity based on functional assays in yeast

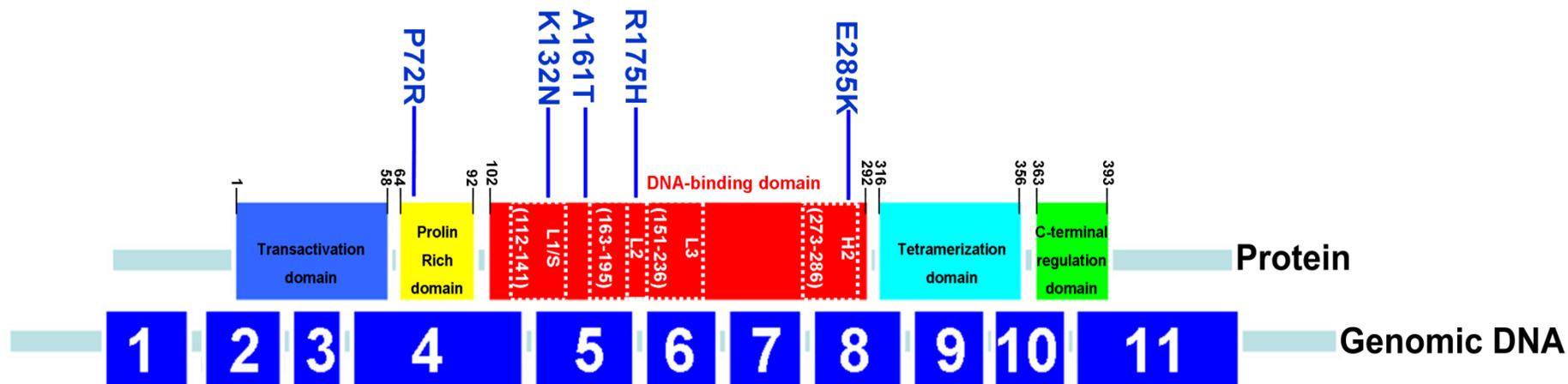


Figure 12. Schematic presentation of TP53 gene and protein. The scheme shows exons, protein domains and missense mutations (shown in blue) detected in myeloma cell lines as well as their locations on the protein domains. The human p53 protein contains 393 codons, which are commonly divided into 5 domains: an RNA polymerase trans-activating domain, a proline rich domain, a DNA-binding domain (the core domain), a tetramerization domain, and a carboxyl-terminal regulatory domain. The structure of the core domain consists of a β sandwich that serves as scaffold for two large loops (L2 (residues 163-195), L3 (residues 236-251)) and loop-sheet-helix region (LSH) (L1 (residues 113-132), S2-S2' (residues 124-135), S10 (residues 264-274) and H2 helix (residues 272-287)). L1, L3 loops and H2 helix form the DNA binding surface of p53 (Adapted from (Cho, Y. et al., Science, 1994), (Whibley, C. et al., Nature review, 2009))

III. Transient siRNA and stable shRNAmir mediated silencing suppresses specifically and efficiently MAGE-C2/CT10 expression in Myeloma cell lines

To investigate, *in vitro*, the biological role of MAGE-C2 in MM, we first transiently transfected 6 Myeloma cell lines with three different MAGE-C2 siRNA, two scrambled siRNA and a FITC labeled non-specific siRNA oligo for the assessment of the efficiency of siRNA transfection.

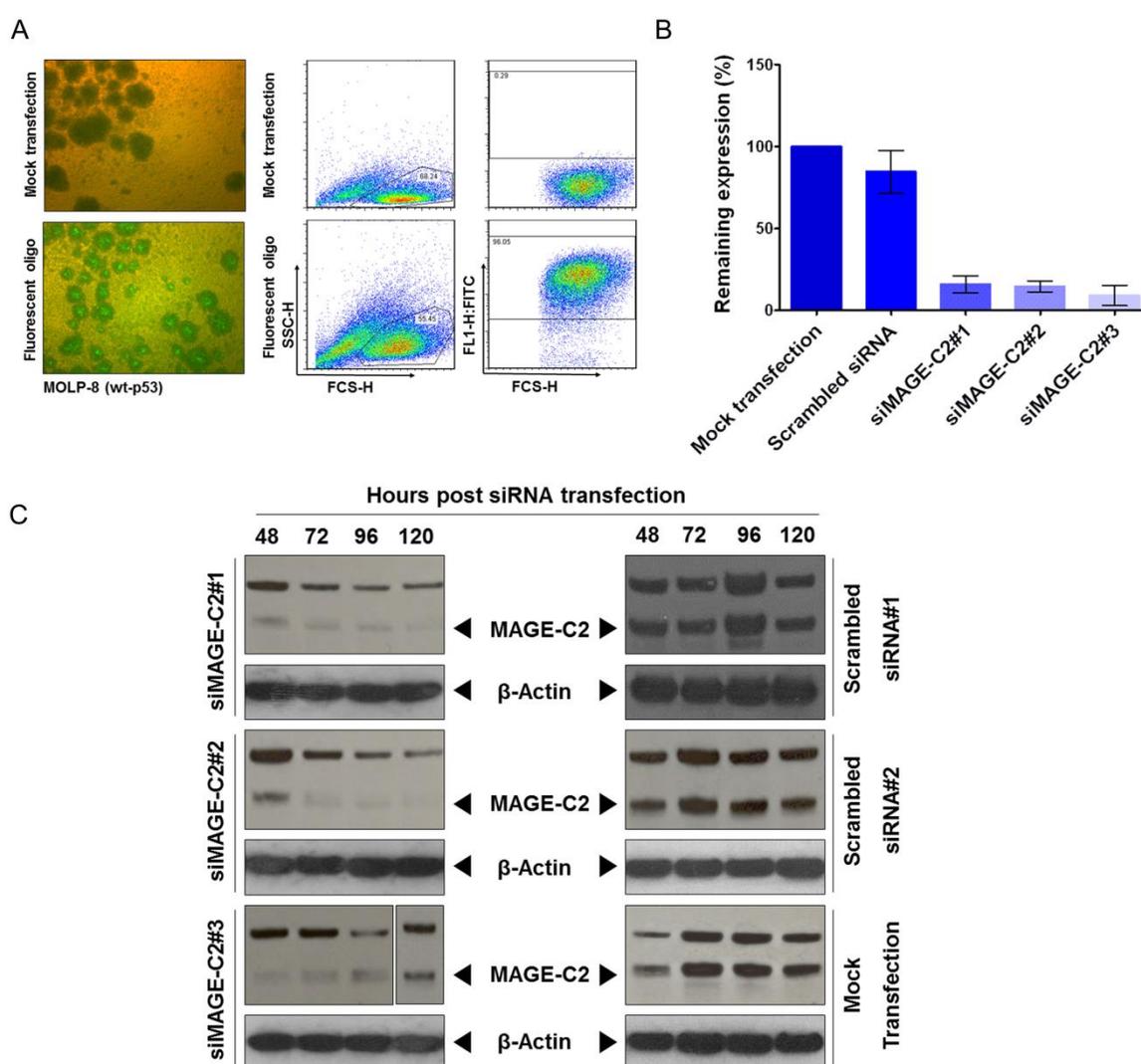


Figure 13. Transient siRNA mediated silencing of MAGE-C2/CT10 in MOLP-8 myeloma cell line. (A) MOLP-8 (wt-p53) was transfected with three different siRNA targeting MAGE-C2 mRNA, 2 scrambled siRNA and FITC labeled non-specific siRNA oligo. At 24h, the efficiency of siRNA uptake was assessed by fluorescence microscopy (Carl Zeiss, Magnification x10) and by FACS. (B) MAGE-C2 knockdown efficiency was assessed by quantitative RT-PCR at 48h post-transfection. The graph

depicts the remaining expression of MAGE-C2 mRNA in myeloma cells transfected with MAGE-C2 siRNA, normalized to the expression of GAPDH housekeeping gene and relative to the expression of MAGE-C2 mRNA in Myeloma cells treated only with Lipofectamine 2000 (Mock transfection). (C) Western blot analysis of MAGE-C2 knockdown Kinetic in MOLP-8 was performed with whole cell lysate harvested at 48, 72, 96 and 120 post-transfection. β -Actin served as a loading control.

We achieved a transfection efficiency of 95% and a selective down-regulation of MAGE-C2 mRNA at 24h and 48h post-transfection (Figure 13A, B). Analysing knock-down kinetics showed optimal MAGE-C2 knockdown observed at 3-4 days post-transfection, while this effect was not observed with the scrambled siRNA constructs as well as Mock transfection (Figure 13C).

Since not all myeloma cell lines showed an efficient siRNA uptake and robust MAGE-C2 knockdown with reduced off-target effects, we manipulated MAGE-C2 expression with constitutive and conditional stable shRNA lentiviral silencing. For the stable loss of function, we used lentiviral micro-RNA based vectors expressing constitutively under the pol II CMV-based promoter, a bi-cistronic transcript encoding for the green fluorescent protein (GFP) and short hairpin RNA (shRNA) cloned within microRNA30 (mir) backbone (shRNAmir). Because we hypothesised an anti-apoptotic function for MAGE-C2, whose inactivation might lead to the loss of cell viability, we also applied a conditional RNAi-mediated gene silencing by using two IPTG-responsive lac repressor-operator mediated inducible shRNA lentiviral vectors expressing under the control of a modified pol III human U6-based promoter. The packaging of replication deficient lentiviral particles was determined using HEK-293T cells. The average titer of the produced viral particles, the functional titer and the relative transduction efficiency in myeloma cell lines are summarized in Figure 14 and Figure 15.

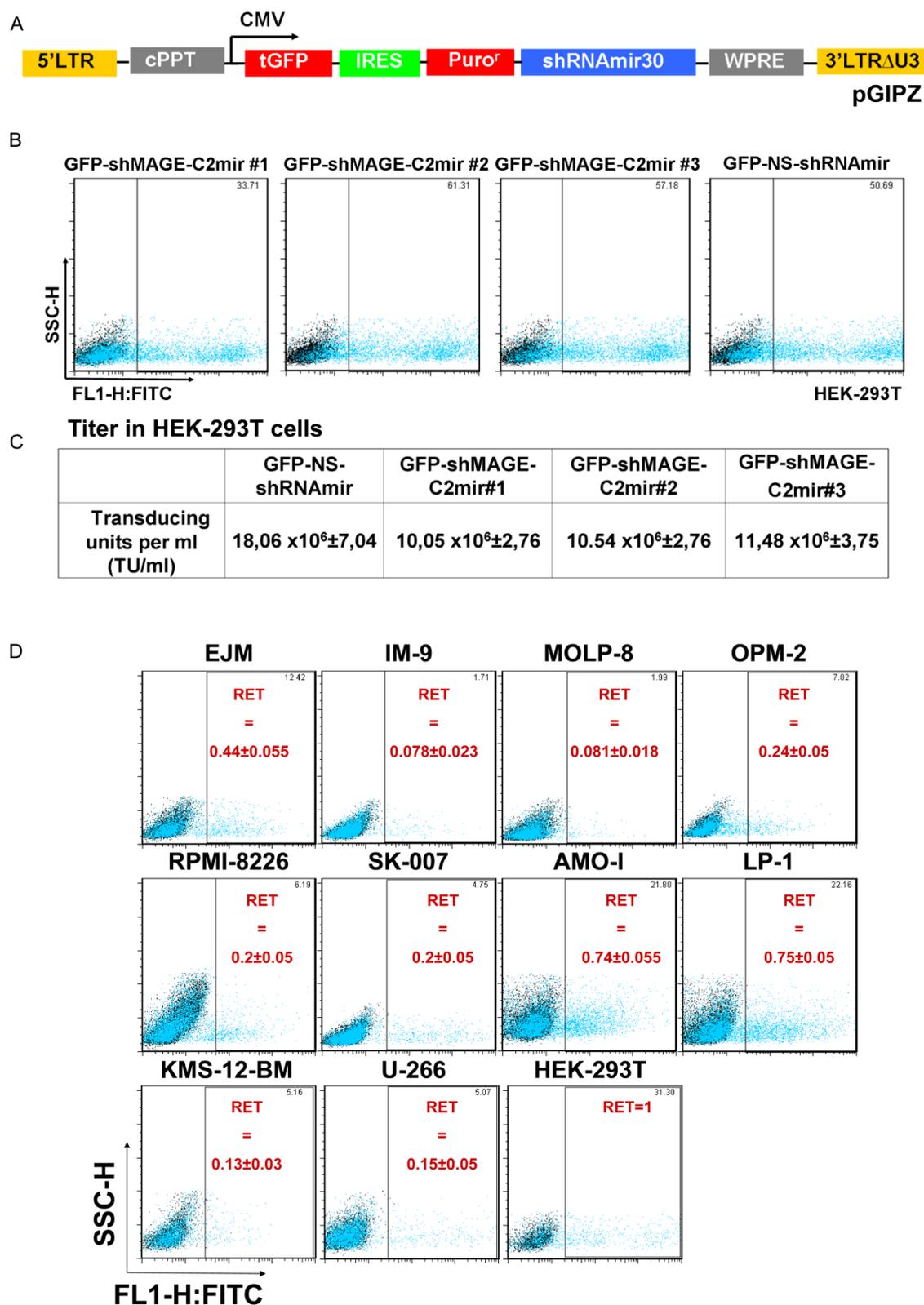


Figure 14. GFP-shRNAmir Lentiviral vector production. (A) Schematic presentation of GFP-shRNAmir (pGIPZ) lentiviral vectors used for MAGE-C2 silencing. (B) Transduction efficiency of HEK-293T cells 48h post-infection with GFP-NS-shRNAmir. (C) The biological titer was calculated according to the following formula: TU (transducing units)/ml = $P \times N / V$, where P = % GFP+ cells, N = number of HEK-293T cells at time of transduction (5×10^4) and V = volume of dilution added to each

well. **(D)** The transduction efficiency of myeloma cell lines 48h post-infection with GFP-NS-shRNAmir. The functional titer was calculated according to the following formula $TU \text{ (transducing units)/ml} = P \times N / V$, where $P = \% \text{ GFP}^+$ cells, $N = \text{number of myeloma cells at time of transduction (} 5 \times 10^4 \text{)}$ and $V = \text{volume of dilution added to each well}$. The relative efficiency of transduction (RET) of myeloma cell lines were calculated according to the following formula: $\text{Functional titer of Non-silencing control virus in myeloma cell line} / \text{Titer of Non-silencing control virus in HEK293T}$.

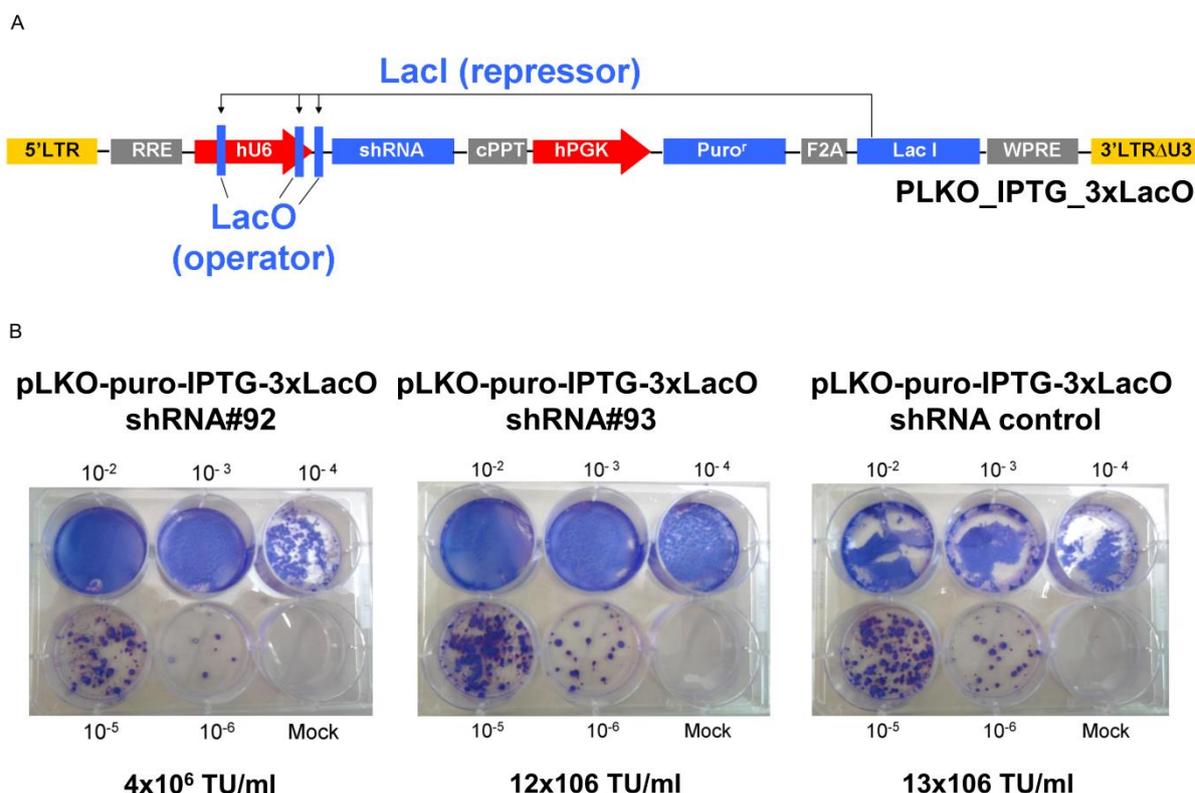


Figure 15. PLKO_IPTG_3xLacO Lentiviral vector production. **(A)** Schematic presentation of IPTG-responsive lac repressor-operator inducible shRNA (PLKO_IPTG_3xLacO) lentiviral vectors used for MAGE-C2 silencing. **(B)** pLKO-puro-IPTG-3xLacO viral particles not expressing any reporter gene were used to transduce HEK-293T cells at different dilution and transduced cells were selected with puromycin (1ug/ml) (Sigma-Aldrich) at 48h post-transduction. After 10 days of selection, stably transduced cells were stained with 1% crystal violet solution and counted for the number of stained colonies.

For this purpose all myeloma cell lines were transduced with three different GFP-shMAGE-C2mir constructs, two IPTG inducible shMAGE-C2, GFP-Non-Specific-shRNAmir and IPTG inducible Non-Specific shRNA constructs at an MOI 20 (MOLP-8 and IM-9), 15 (U-266, SK-007) and 10 (EJM, RPMI-8226, OPM-2). At 48h post-transduction, the percentage of myeloma cells expressing GFP-shRNAmir was assessed by FACS and was about 85 to 95 % in all cell lines (Figure 16).

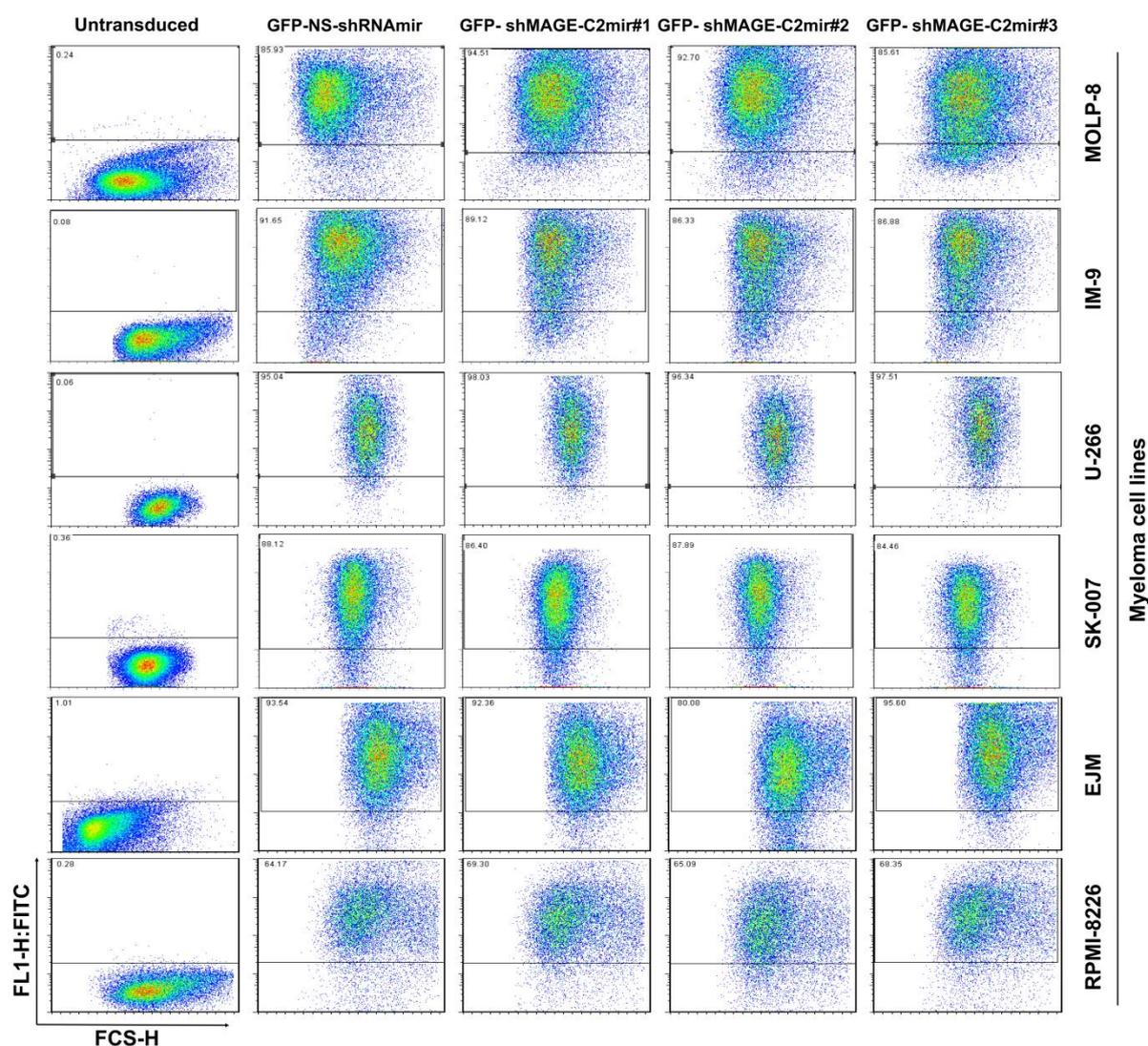


Figure 16. Flow cytometry analysis of the transduction efficiency of GFP-shRNAmir transduced myeloma cell lines.

The validation of MAGE-C2 knockdown at 72h post-transduction by quantitative RT-PCR and Western blot showed that MAGE-C2 silencing was induced in all cells expressing stably and constitutively GFP-shMAGE-C2mir by more than 80% of MAGE-C2 mRNA and protein at 72 hours post-transduction (Figure 17B, C).

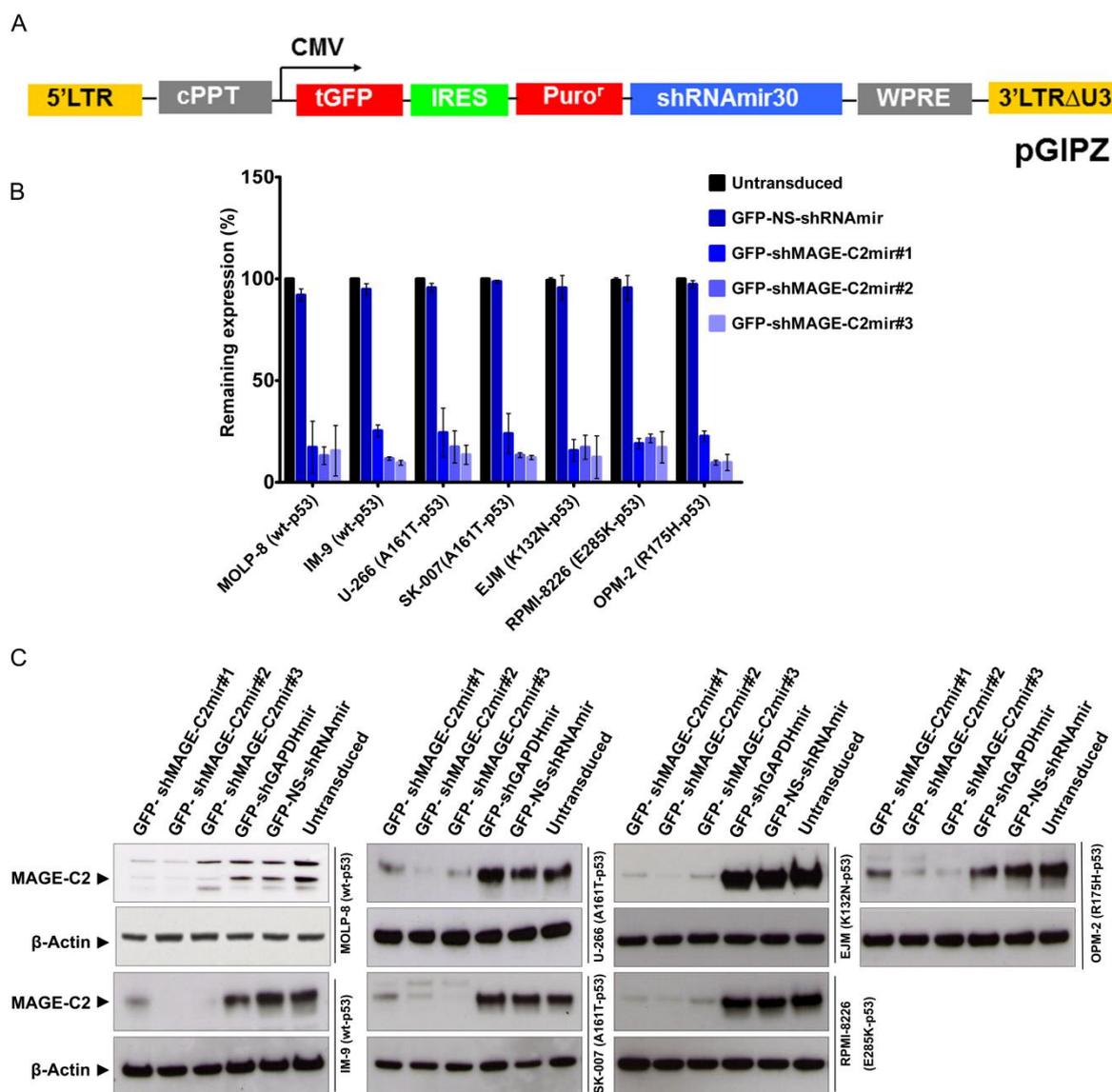


Figure 17. Stable shRNA lentiviral mediated silencing of MAGE-C2/CT10 in myeloma cell lines. (A) Schematic presentation of GFP-shRNAmir lentiviral vectors used for MAGE-C2 silencing. (B) MAGE-C2 silencing mediated by GFP-shRNAmir lentiviral vector in MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53), SK-007 (A161T-p53), EJM (K132N-p53) and RPMI-8226 (E285K), OPM-2 (R175H) was assessed by quantitative RT-PCR at 48h post-infection. (C) Western blot analysis showing the efficiency of MAGE-C2 silencing at 72h post-infection. β -Actin served as a loading control.

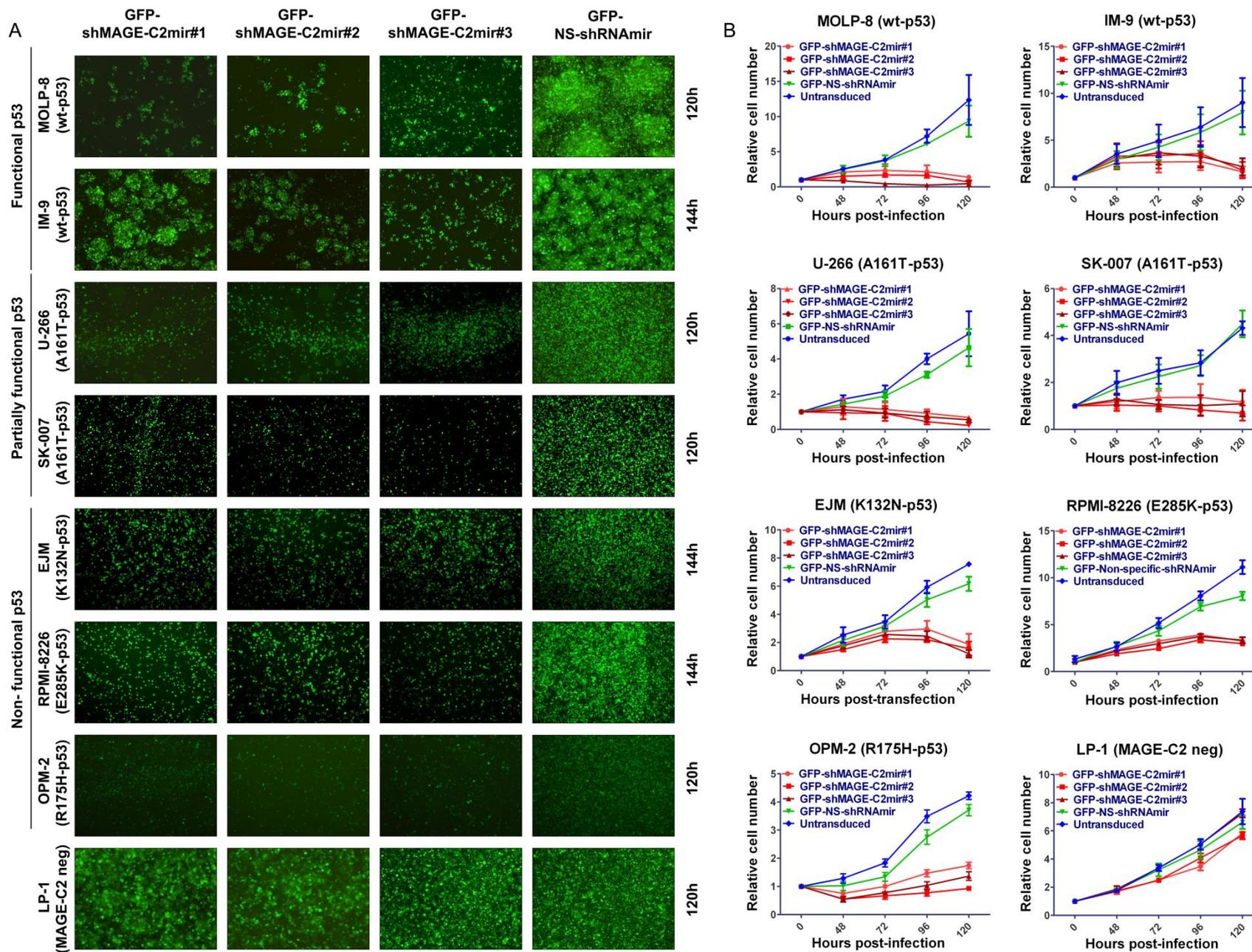
These results confirmed that the MAGE-C2 transient siRNA silencing was not an off-target effects rather MAGE-C2 specific. However, both IPTG-responsive lac repressor-operator mediated inducible shRNA lentiviral vectors was not as potent as GFP-shMAGE-C2mir in inducing MAGE-C2 silencing likely due to the inefficiency of the modified human U6 promoter. These results showed that unlike, transient siRNA and IPTG inducible shRNA mediated silencing, constitutive expression of lentiviral GFP-shRNAmir stably integrated in

myeloma cell lines suppressed specifically and efficiently MAGE-C2 expression with reduced off-target effects; therefore, we performed the following functional analysis only with the stable GFP-shRNAmir lentiviral silencing.

IV. MAGE-C2/CT10 promotes proliferation and resistance to apoptosis in Multiple Myeloma

IV.1 MAGE-C2/CT10 silencing inhibits Myeloma cell growth and viability in vitro

The usage of a bi-cistronic GFP-shRNAmir expression cassette allowed us to track the growth and viability of myeloma cells expressing concomitantly shMAGE-C2mir and the reporter gene GFP over 4 days post-infection by phase inverted/fluorescence microscopy. We found that the lentivirus-mediated silencing of MAGE-C2 significantly slowed down the growth of myeloma cells and suppressed their viability irrespective of p53 mutational status (Figure 18A, B). Conversely, MAGE-C2 depletion had practically no effect on the growth and viability of myeloma cell line LP-1 myeloma that does not express MAGE-C2. Aiming at understanding the molecular mechanisms behind the loss of cell growth and viability induced by MAGE-C2 silencing, we used BrdU incorporation and MTT colorimetric assays, which assess DNA replication of cycling cells and the metabolic activity of cells, respectively. We observed that the decrease of cell growth was due to a decrease in the DNA replication (Figure 18C) as well as a decrease in the viability of all Myeloma cell lines expressing shMAGE-C2mir (Figure 18D), suggesting an important role of MAGE-C2 in promoting the proliferation and the survival of myeloma cells irrespective of the functional status of p53.



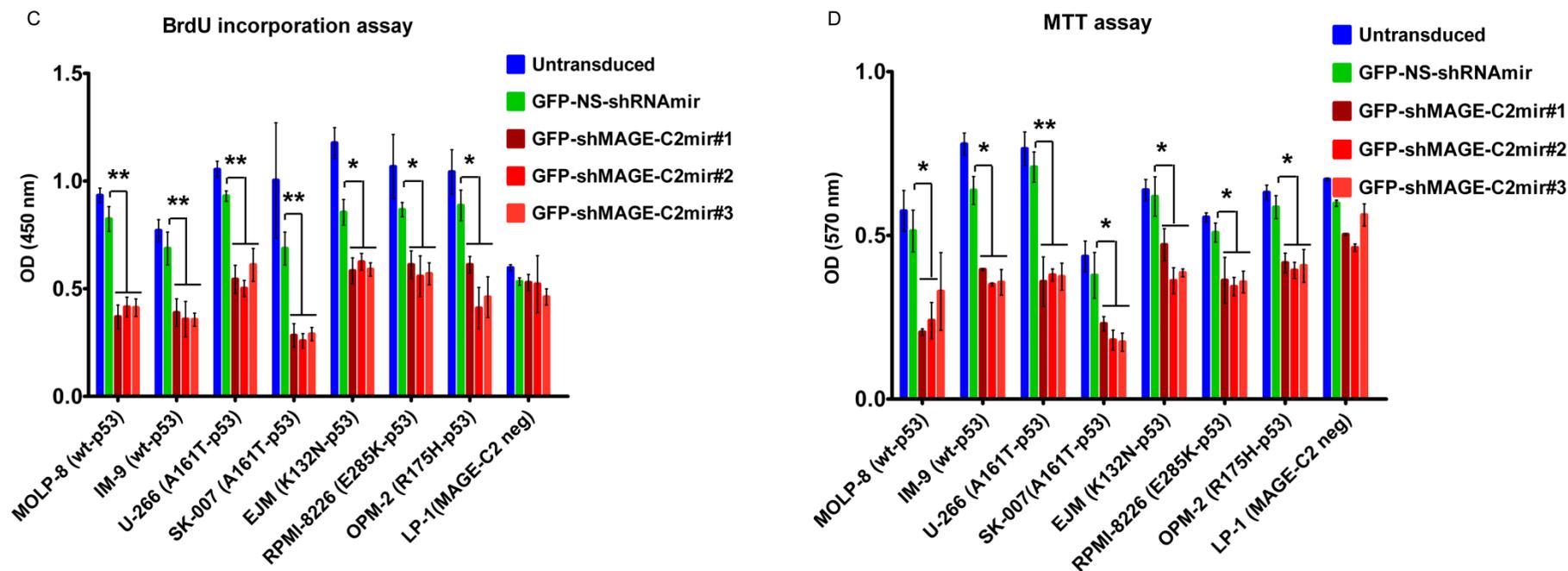


Figure 18. MAGE-C2/CT10 silencing inhibits myeloma cell proliferation and viability in vitro. The growth and viability of MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53), SK-007 (A161T-p53), EJM (K132N-p53) and RPMI-8226 (E285K), OPM-2 (R175H) transduced with three GFP-shMAGE-C2mir vectors and the GFP-Non-specific-shRNAmir control vector were monitored over 6 to 7 days by phase contrast/ fluorescence microscopy (Carl Zeiss) and by counting cells that excluded Trypan blue dye. (A) Snapshots of myeloma cells in culture expressing GFP-shRNA-mir were acquired at 120h or 144h post-infection by AxioCam Camera (Magnification x10). (B) The growth curves represent the relative cell number corresponding to cell number at each time point post-transduction (48, 72, 96 and 120h) normalized to the starting cell number. (C) Cell proliferation was measured by BrdU proliferation assay at 96h post-infection. (D) Cell viability was assessed by MTT assay at 120h (MOLP-8, U-266, SK-007, OPM-2) or 144h (IM-9, EJM, RPMI-8226) post-infection. Data at each time point represents the mean of three independent experiments performed each one in triplicate and error bars present standards deviations from the mean. Statistical significance was accepted with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells.

IV.2 MAGE-C2/CT10 silencing impairs cell cycle progression and induces apoptosis in Myeloma cells

To further define mechanisms behind the anti-proliferative effect of MAGE-C2/CT10 silencing, we analysed kinetics of cell cycle progression of GFP-shMAGE-C2mir-expressing myeloma cells by flow cytometry. We found, that MAGE-C2 depletion in myeloma cells expressing fully functional (IM-9, MOLP-8), partially functional (U-266, SK-007) and non-functional (EJM, RPMI-8226) p53 impaired cell cycle progression at both G1/S and/or G2/M transition (Figure 19, Figure 20, Figure 21, Figure 22, Figure 23, Figure 24, Figure 25). MAGE-C2 silencing induced a slight increase in the G0/G1 population at 48h in EJM (K132N-p53), at 72h in IM-9(wt-p53), U-266 (A161T-p53), and at 96h in MOLP-8 (wt-p53), SK-007(A161T-p53) and RPMI-8226 (E285K-p53). A more pronounced effect was observed for the G2/M transition where MAGE-C2 silencing induced 2-3 fold increase in all cell lines except EJM. Moreover, we found that the anti-proliferative effect of MAGE-C2 depletion was due to a 50-70% decrease of cells in the S phase of the cell cycle. These data indicate that the anti-proliferative effect of MAGE-C2 silencing is due to an impairment of cell cycle progression through G0/G1 and/or G2/M transition and a decrease in the percentage of cells replicating their DNA in S phase, supporting a role for MAGE-C2 in facilitating cell cycle progression of myeloma cells.

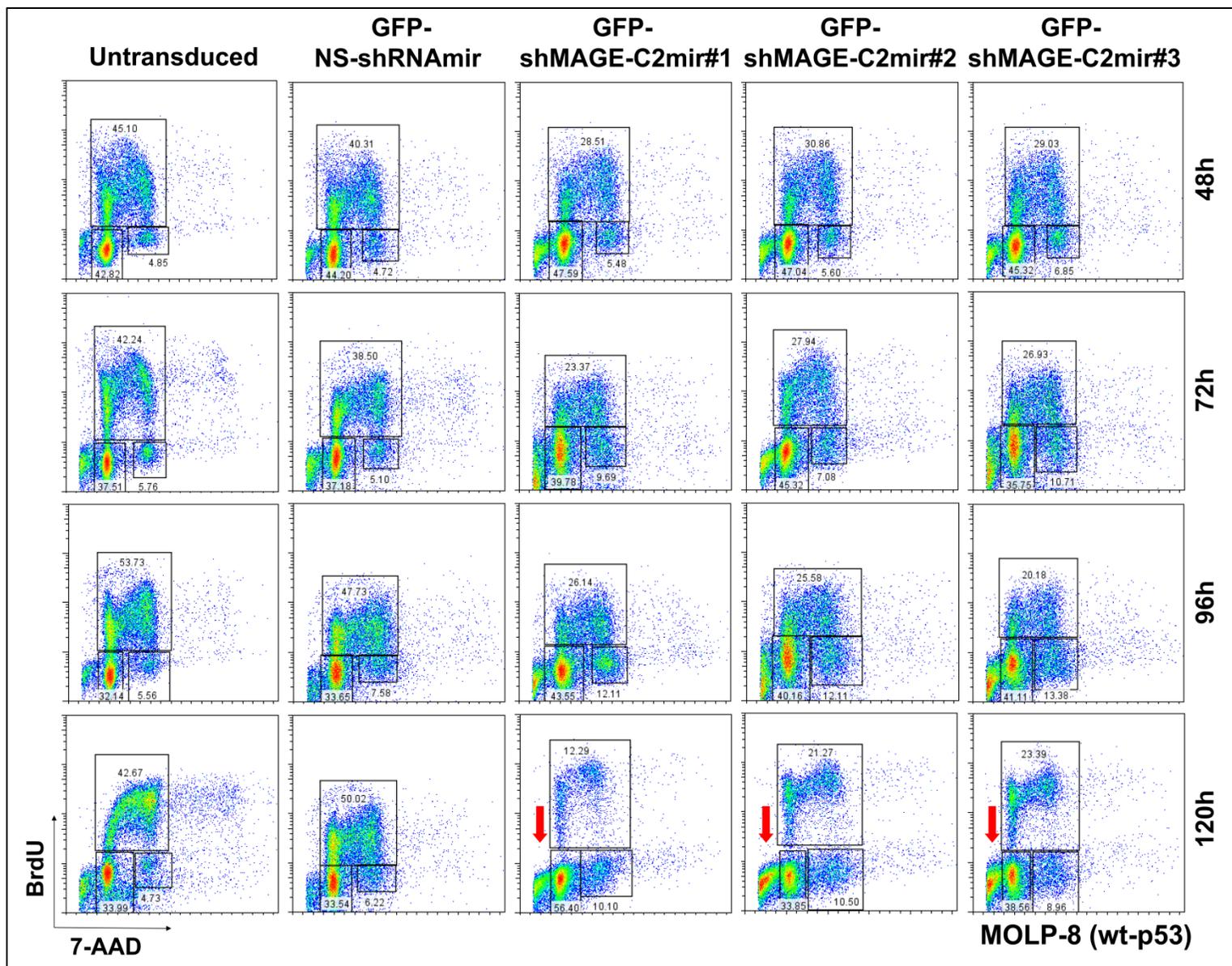


Figure 19. The effect of MAGE-C2/CT10 silencing on cell cycle progression of MOLP-8. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at the G1/S, G2/M phase at 96h and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection

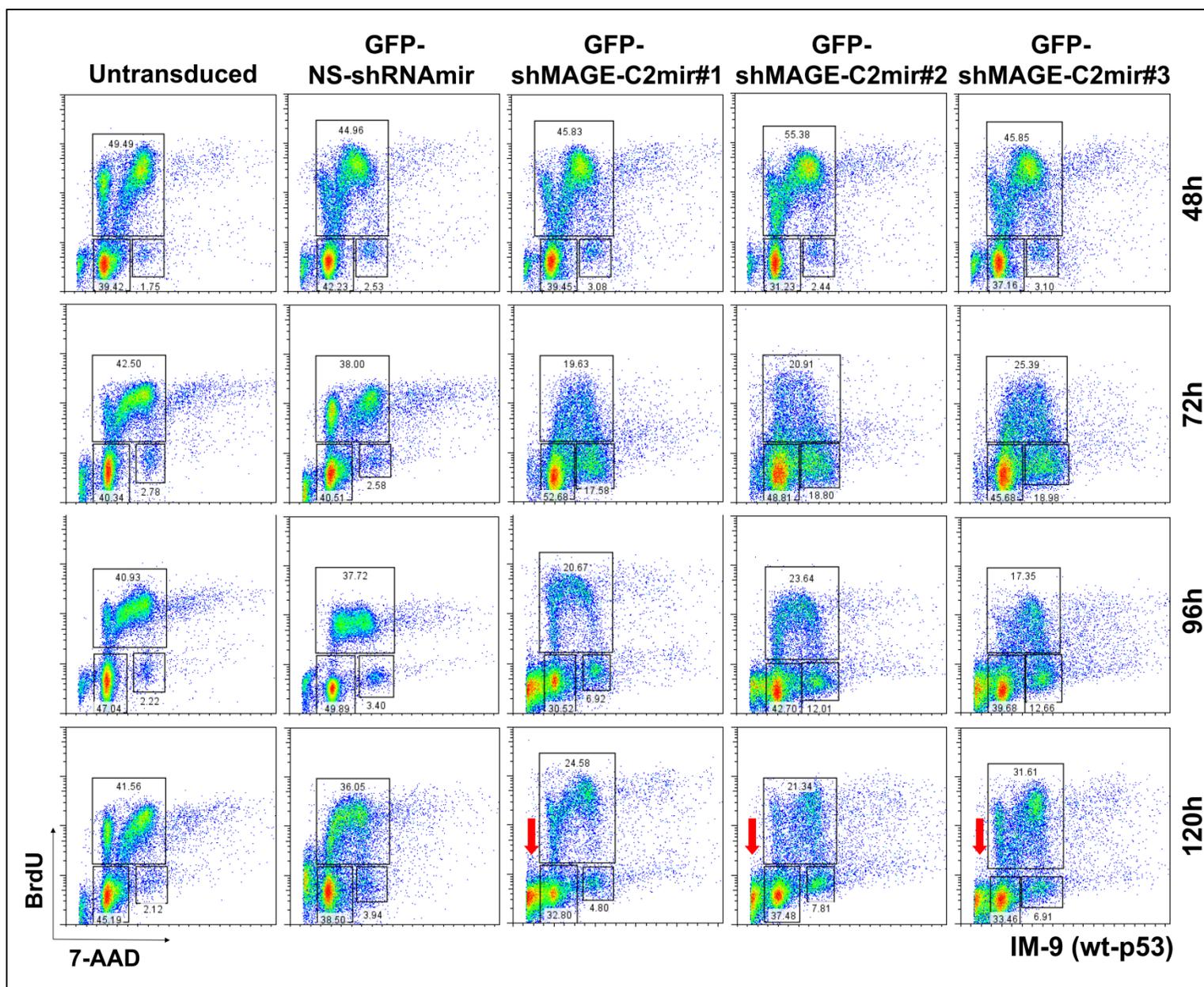


Figure 20. The effect of MAGE-C2/CT10 silencing on cell cycle progression of IM-9. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at the G1/S, G2/M phase at 72h and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection.

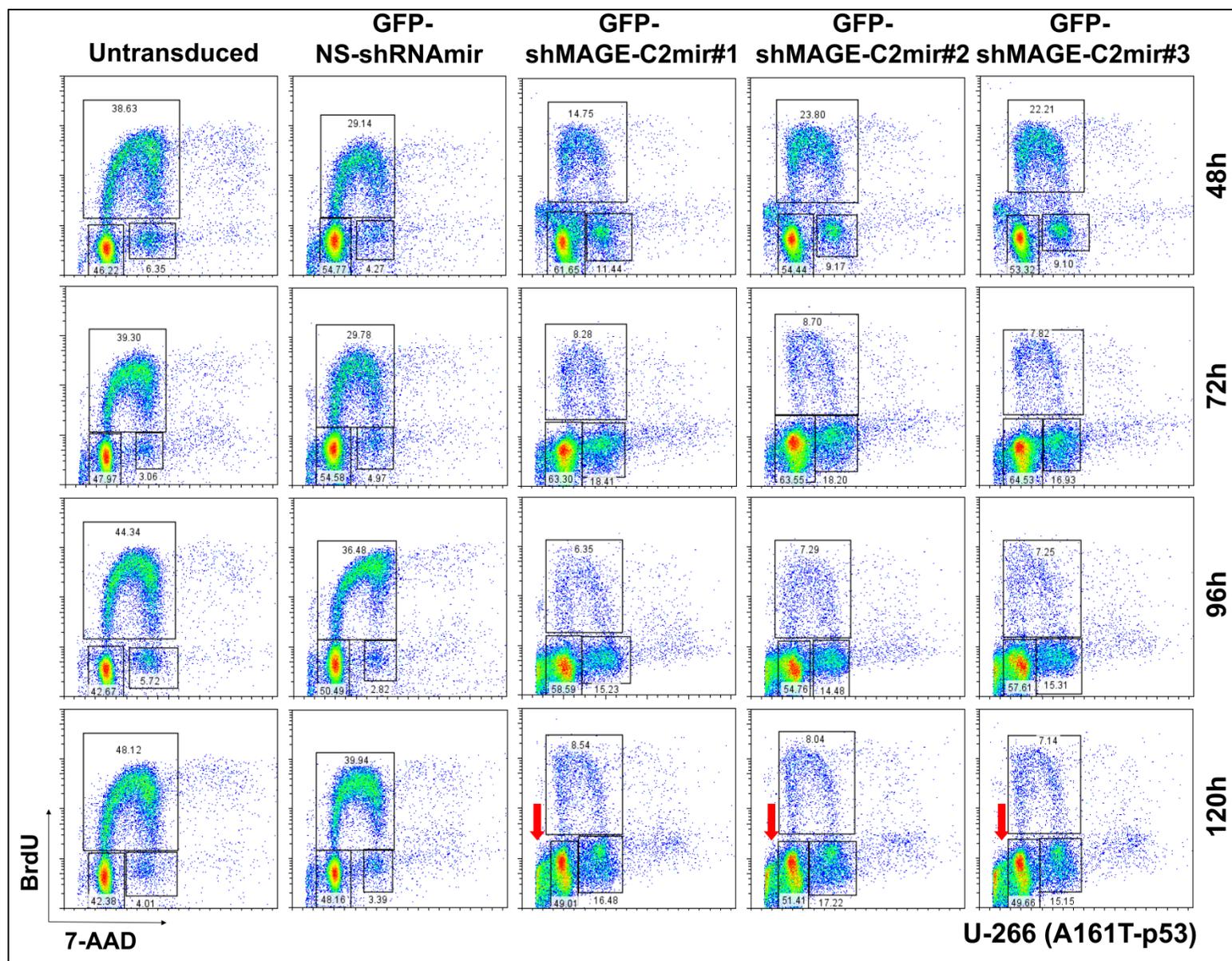


Figure 21. The effect of MAGE-C2/CT10 silencing on cell cycle progression of U-266. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at 72h the G1/S, G2/M phase and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection.

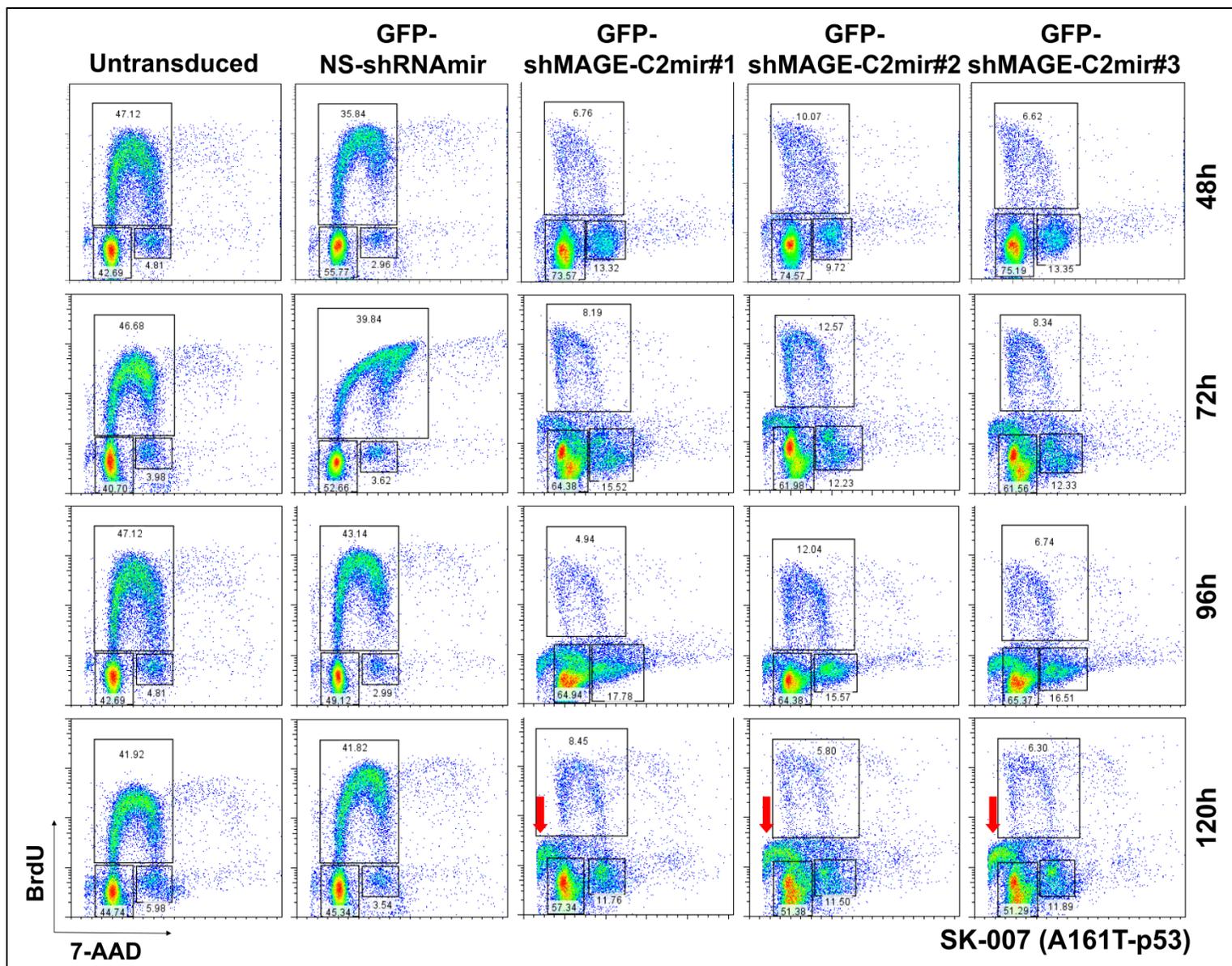


Figure 22. The effect of MAGE-C2/CT10 silencing on cell cycle progression of SK-007. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at 48h the G1/S, G2/M phase and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection.

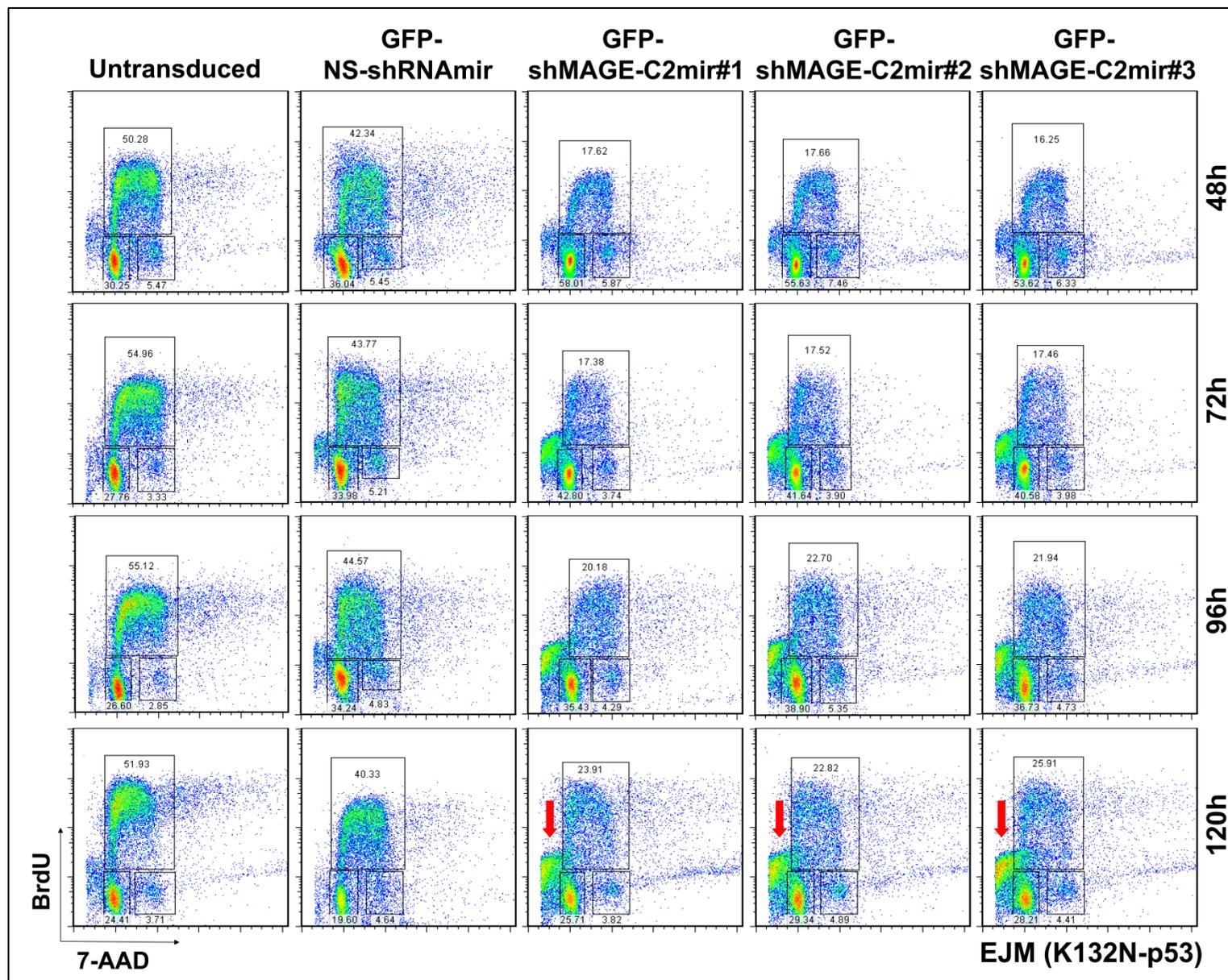


Figure 23. The effect of MAGE-C2/CT10 silencing on cell cycle progression of EJMs. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at 48h the G1/S, G2/M phase and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection.

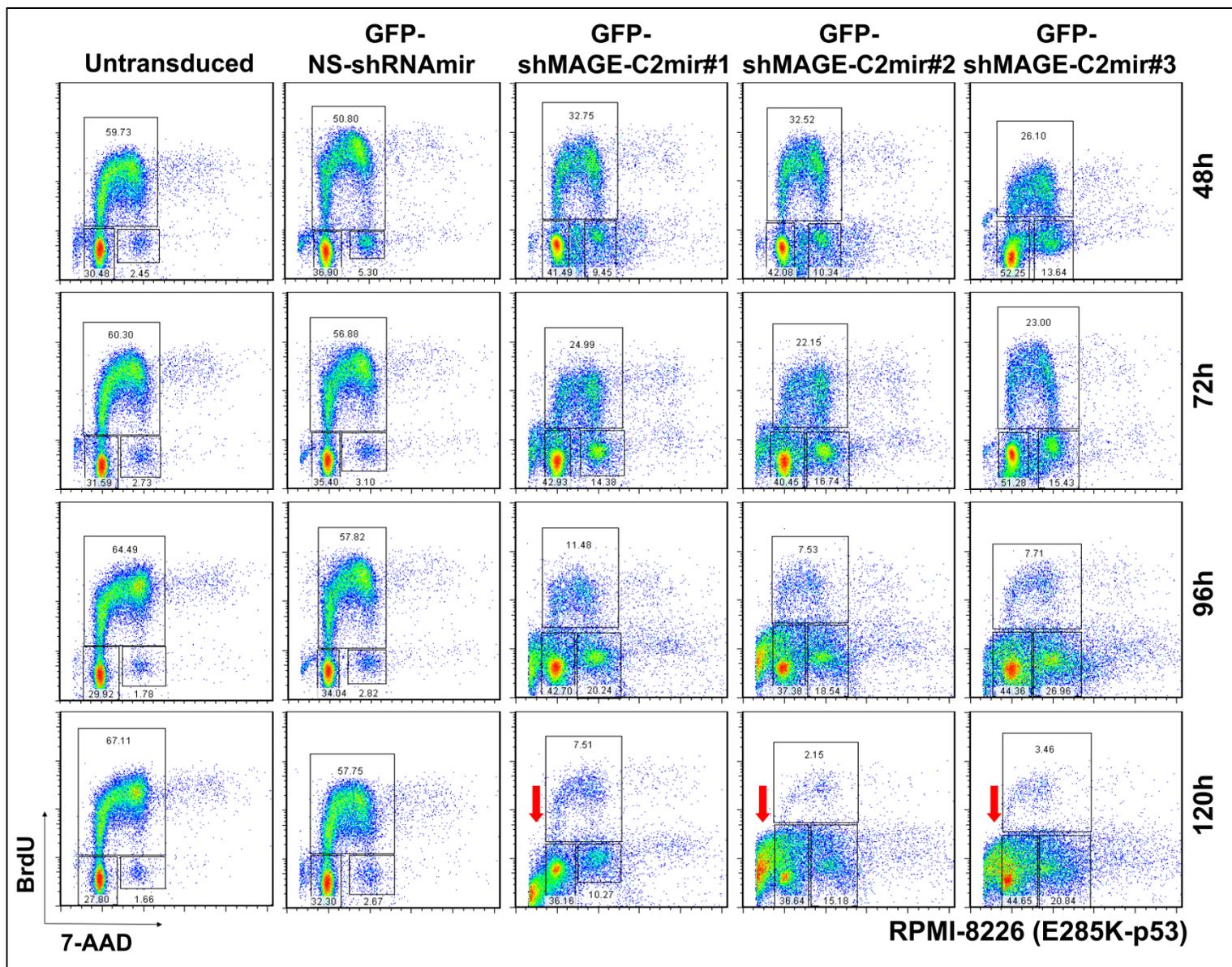


Figure 24. The effect of MAGE-C2/CT10 silencing on cell cycle progression of RPMI-8226. Myeloma cells transduced with GFP-shMAGE-C2mir or GFP-Non-Specific-shRNAmir control vectors were harvested at 48, 72, 96 and 120 h post-infection and analyzed by BrdU and 7-AAD intracellular staining and flow cytometry. The results show that the anti-proliferative effect of MAGE-C2 silencing was due to a decrease in the S phase, an increase at 48h the G1/S, G2/M phase and an increase of the subG0/G1 diploid population (pointed with red arrows) at 120h post-infection

C

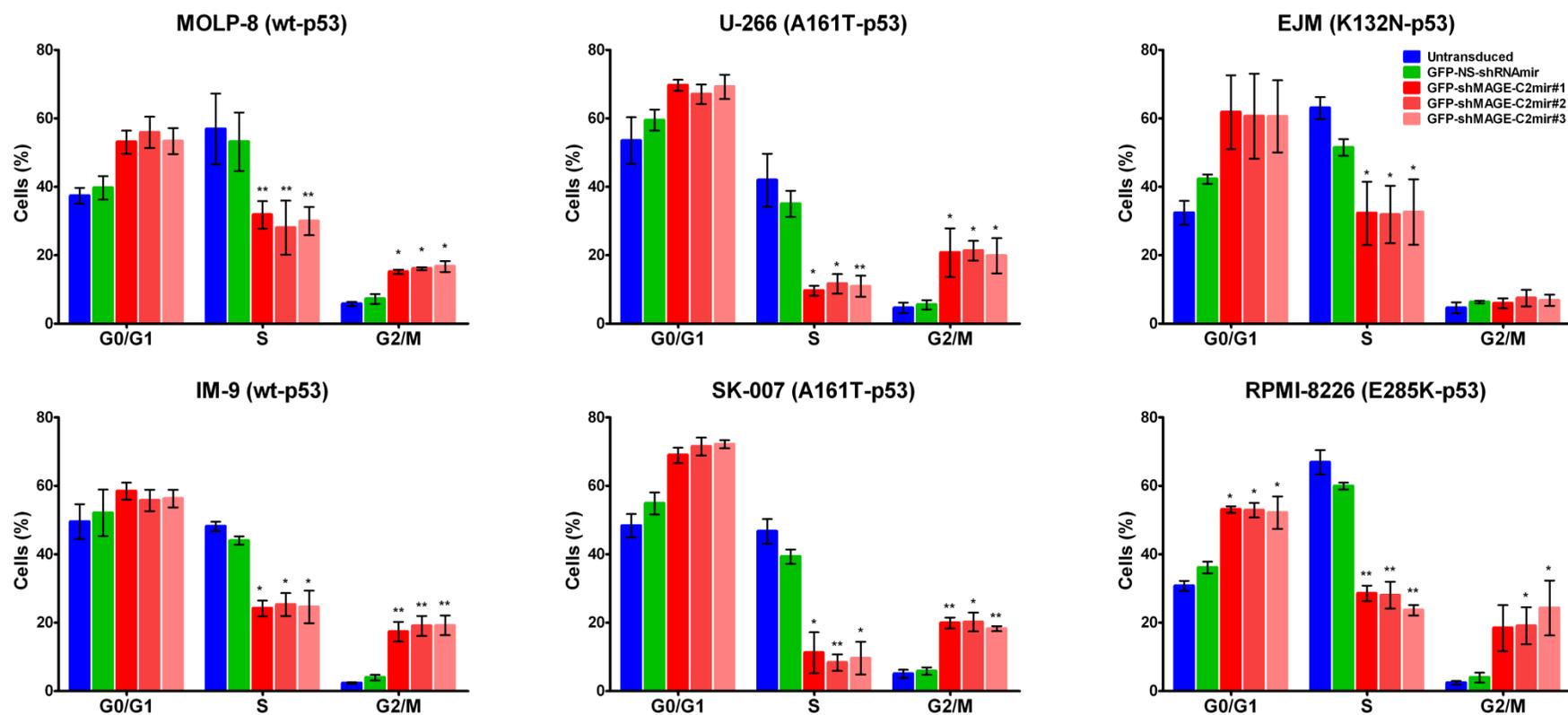


Figure 25. The effect of MAGE-C2/CT10 silencing on cell cycle progression of myeloma cell lines. Histogram bars show the percentage of cells in G0/G1, S and G2/M phases at one time point post-infection (48h (EJM), 72h (IM-9, U-266) or 96h (MOLP-8, SK007 and RPMI-8226)) in untransduced and transduced myeloma cells with GFP-Non-specific-shRNAmir or GFP-shMAGE-C2mir vectors. The results present the mean of three independent experiments and error bars present standard deviations from the mean. Statistical significance was accepted with *P < 0.05, **P < 0.01, ***P < 0.001 comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells.

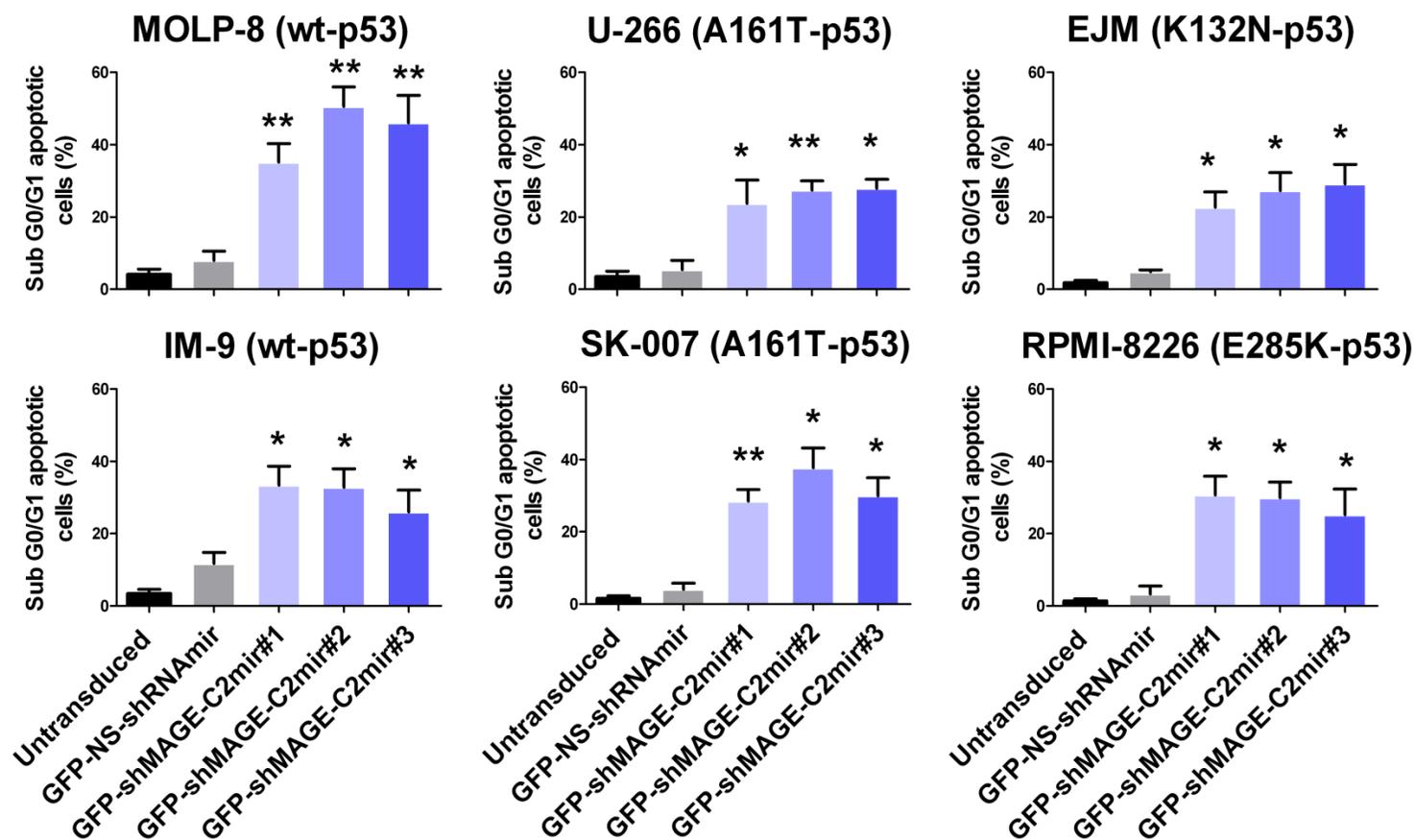


Figure 26. MAGE-C2/CT10 silencing increases the sub-diploid G0/G1 population in myeloma cell lines. Histogram bars show the percentage of subG0/G1 diploid population. The results present the mean of three independent experiments and error bars present standard deviations from the mean. Statistical significance was accepted with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells.

Because the MTT assay had shown a decrease in cell viability following MAGE-C2 silencing and we had observed an increase in the sub-G0/G1 diploid population of the cell cycle (Figure 26), which is indicative of an increased level of apoptosis, we next determined the percentage of apoptotic cells after MAGE-C2 silencing using flow cytometry. These analyses showed that MAGE-C2 knockdown indeed specifically induced apoptosis and cell death as indicated by Annexin and 7-AAD positivity, respectively (Figure 27, Figure 28), indicating a strong anti-apoptotic property of MAGE-C2 in multiple myeloma.

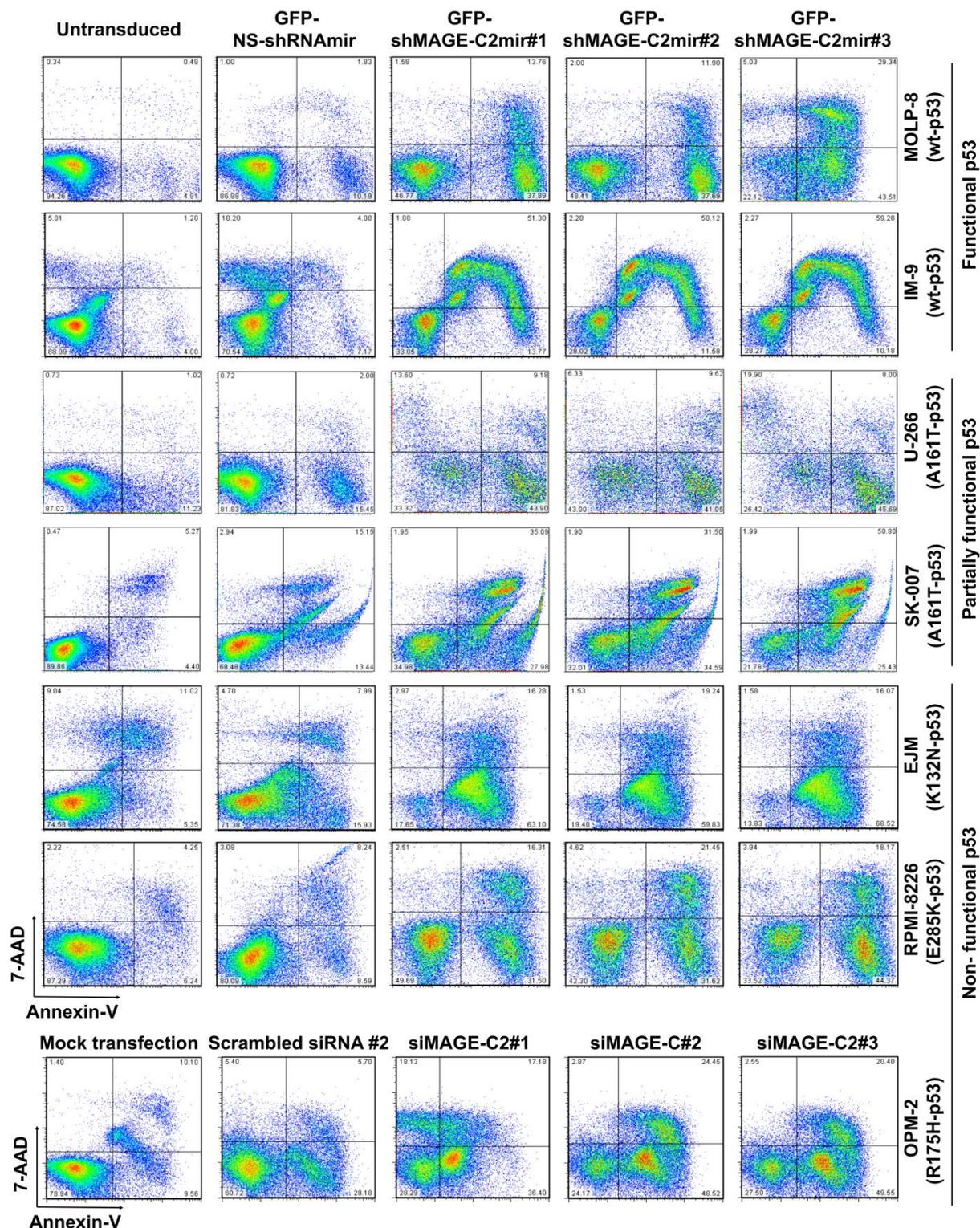


Figure 27. MAGE-C2/CT10 silencing activates apoptotic cell death in Myeloma cell lines. The Analysis of apoptosis by Annexin-V and 7-AAD staining show that MAGE-C2 silencing, by transient siRNA or by stable GFP-shRNAmir based lentivirus activated the apoptotic cell death in all myeloma cell lines expressing function or non-functional p53. (B) Graphs depict the percentage of Annexin positive cells. The results present the mean of three independent experiments and error bars present standards deviations from the mean. Statistical significance was accepted with *P<0.05, **P<0.01, ***P<0.001 comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells and siMAGE-C2 to scrambled siRNA#2.

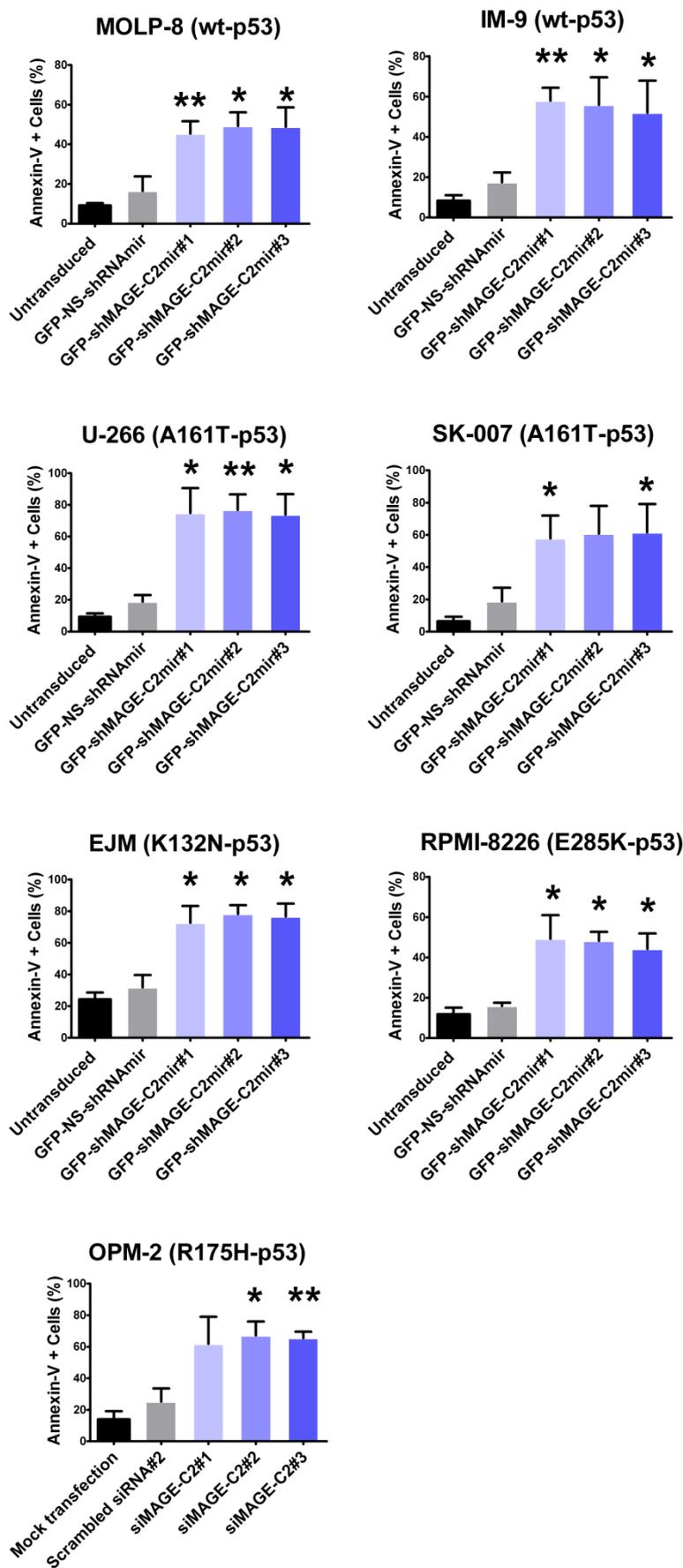


Figure 28. MAGE-C2/CT10 silencing activates apoptotic cell death in myeloma cell lines. Graphs depict the percentage of Annexin positive cells. The results present the mean of three independent experiments and error bars present standards deviations from the mean. Statistical significance was accepted with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells and siMAGE-C2 to scrambled siRNA#2.

IV.3 MAGE-C2/CT10 over-expression rescues the anti-proliferative effect of MAGE-C2/CT10 silencing and protects cells from apoptotic cell death

To confirm the specificity of MAGE-C2 silencing effect on myeloma proliferation and viability, we performed MAGE-C2 silencing along with overexpression of MAGE-C2 lacking the 3'UTR, which conversely to the endogenous MAGE-C2 protein cannot be targeted by the shMAGE-C2mir (Figure 29A).

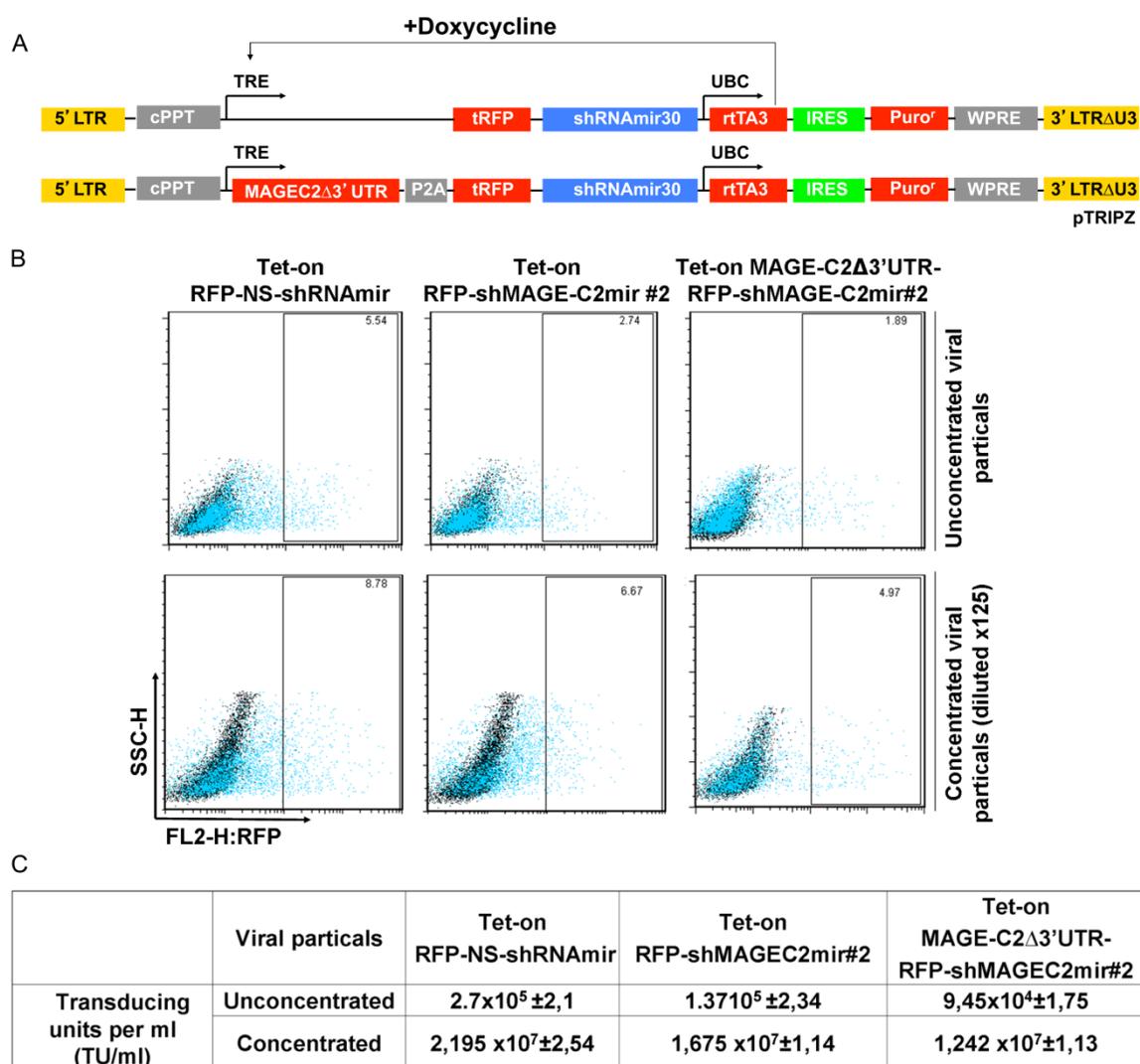
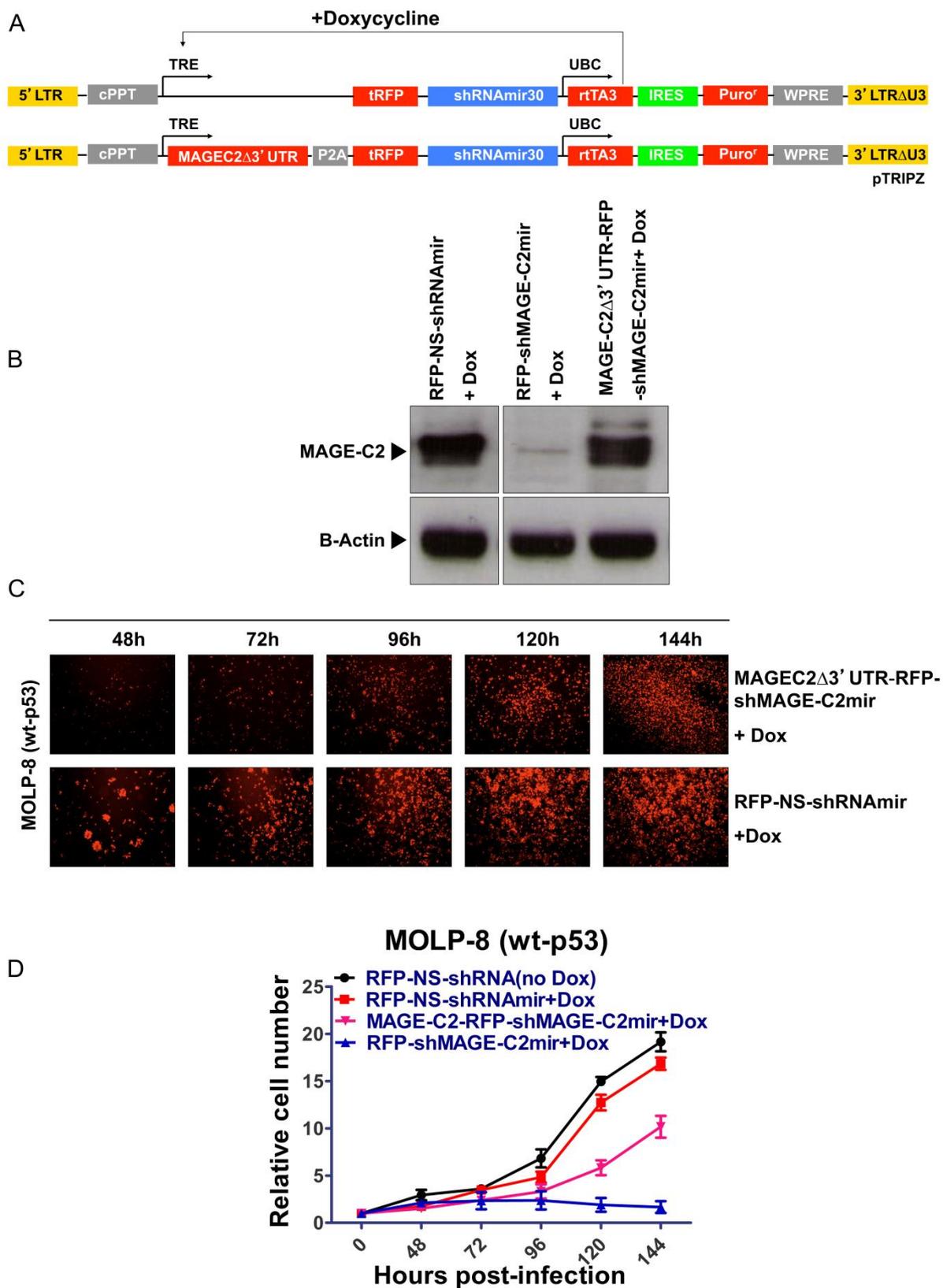


Figure 29. Design and production of Tet-on lentiviral inducible MAGE-C2Δ3'UTR-TurboRFP-shRNAmir lentivirus. (A) Schematic presentation of the Tet-on lentiviral inducible RFP-shRNAmir and MAGE-C2Δ3'UTR-TurboRFP-shMAGE-C2mir used for the MAGE-C2 silencing and rescue, respectively. MAGE-C2Δ3'UTR cDNA was sub-cloned in a doxycycline regulated Tet-on lentiviral inducible RFP-shRNAmir vector at the single AgeI restriction site, upstream and in-frame of the bicistronic TurboRFP-shRNAmir transcript. (B) Transduction efficiency of HEK-293T cells 48h hours

post-infection with unconcentrated and concentrated lentiviral particles (black dot blots present untransduced cells (control population), blue dot blots present transduced cells). (C) The functional titer was calculated according to the following formula $TU (transducing\ units)/ml = P \times N / V$, where $P = \%GFP+$ cells, $N =$ number of myeloma cells at time of transduction (5×10^4) and $V =$ volume of dilution added to each well.

At 96 hours post-doxycycline induction, MOLP-8-transduced cells were harvested and assessed for the efficiency of knockdown by Western blot analysis. We found that, MOLP-8 (wt-p53) cells expressing the MAGE-C2 Δ 3'UTR-RFP-shMAGE-C2mir transcript increased the protein expression of the exogenous MAGE-C2, while Myeloma cells expressing only RFP-shMAGE-C2mir showed robust suppression of MAGE-C2 protein expression. We found that MAGE-C2 overexpression restored the knockdown phenotype and at least partially reversed the anti-proliferative effect of MAGE-C2 silencing. Interestingly, despite the delay of cell cycle progression at the G2/M boundary, there was no increase in the subG0/G1 diploid population suggesting that MAGE-C2 overexpression rescued MAGE-C2 silencing phenotype by facilitating the progression through the cell cycle and conferring resistance to apoptotic cell death. These findings indicate that MAGE-C2 exerts its anti-apoptotic function in myeloma at least partly through the regulation of genes controlling cell cycle progression and apoptosis (Figure 30).



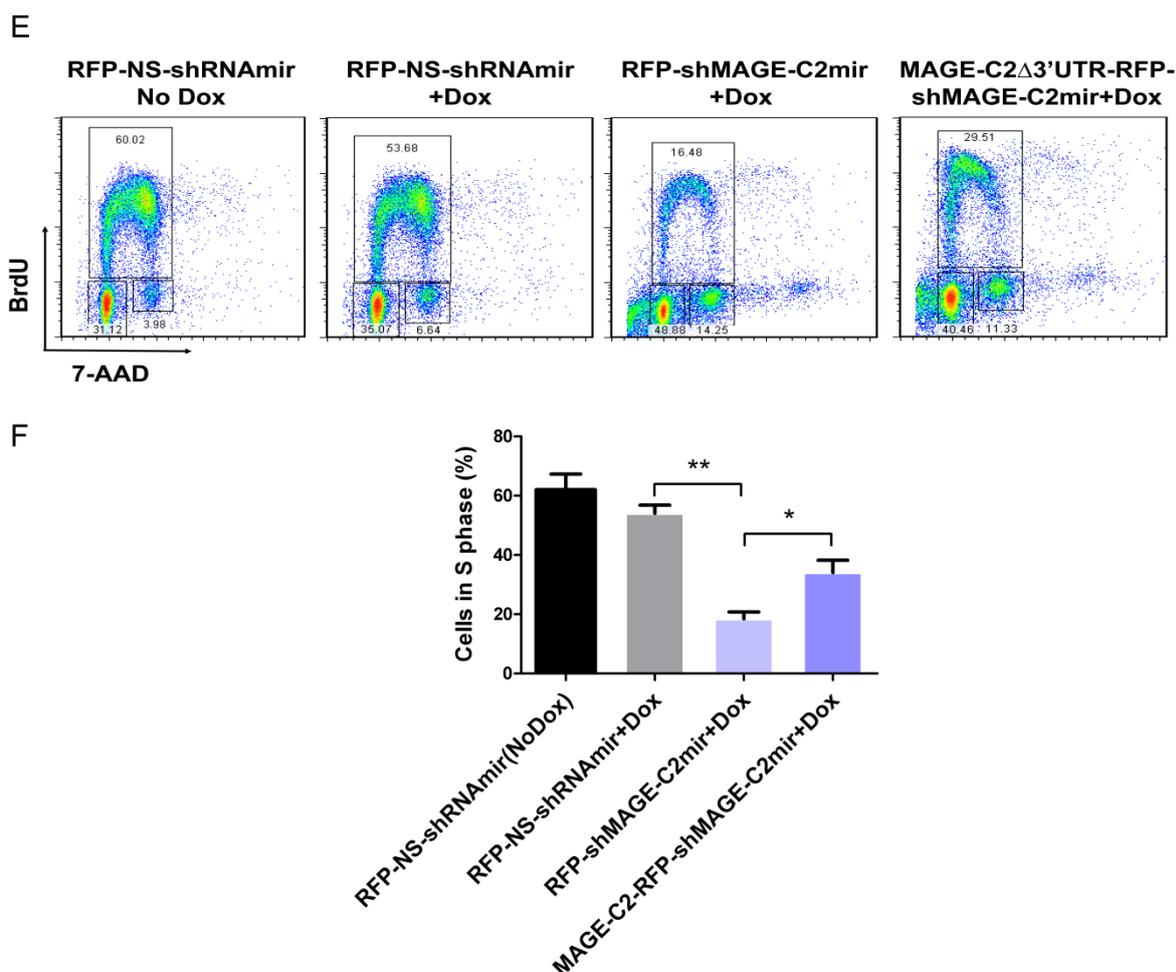


Figure 30. MAGE-C2/CT10 over-expression rescues the anti-proliferative effect of MAGE-C2/CT10 silencing and protects cells from apoptotic cell death. (A) Schematic presentation of the Tet-on lentiviral inducible RFP-shRNAmir and MAGE-C2 Δ 3'UTR-TurboRFP-shMAGE-C2mir used for the MAGE-C2 silencing and rescue, respectively. (B) Western blot analysis of whole cell lysate harvested 96h post-doxycycline induction shows an efficient silencing of MAGE-C2 silencing with TurboRFP-shMAGE-C2mir vector, while MAGE-C2 Δ 3'UTR-RFP-shMAGE-C2mir vector restored MAGE-C2 expression. (C) The growth and viability of MOLP-8 (wt-p53) myeloma cells transduced cells with MAGE-C2 Δ 3'UTR-RFP-shMAGE-C2mir and RFP-Non-specific-shRNAmir and treated with doxycycline were assessed by phase-contrast/fluorescence microscopy. (D) Counting cells that excluded trypan blue exclusion dye over 144h post-doxycycline induction showed that MAGE-C2 Δ 3'UTR overexpression rescued the anti-proliferative effect of MAGE-C2 silencing. (E) The analysis of cell cycle distribution shows that MAGE-C2 Δ 3'UTR overexpression facilitated cell cycle progression in comparison to the effect of MAGE-C2 silencing and protected cells from apoptotic cell death. (F) Histogram bars show the percentage of cells in the S phase. The results present the mean of three independent experiments and error bars present standard deviations from the mean. Statistical significance was accepted with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing RFP-shMAGE-C2mir transduced cells to RFP-Non-Specific-shRNAmir transduced cells and MAGE-C2 Δ 3'UTR-RFP-shMAGE-C2mir to RFP-shMAGE-C2mir.

V. MAGE-C2/CT10 silencing inhibits the anchorage-independent growth of Myeloma cells

Apoptosis resistance is a hallmark of clonogenic Myeloma stem cells, which are thought to confer chemotherapy resistance and relapse of myeloma patients initially responding to treatment [132]. It has been indicated that progenitor cells are also present in myeloma cell lines and that these cells can be identified in the bulk culture based on their anchorage-independent growth ability to form colonies in semi-solid medium [132, 290]. The analysis of the clonogenic property of myeloma cell lines depleted of MAGE-C2 expression showed a specific and significant decrease in the size and number of colonies likely due to a decrease in cell proliferation and an increase of cell death. This effect was independent of the p53 mutational status (Figure 31, Figure 32). These findings indicate a role of MAGE-C2 in maintaining the clonogenic property of myeloma cells.

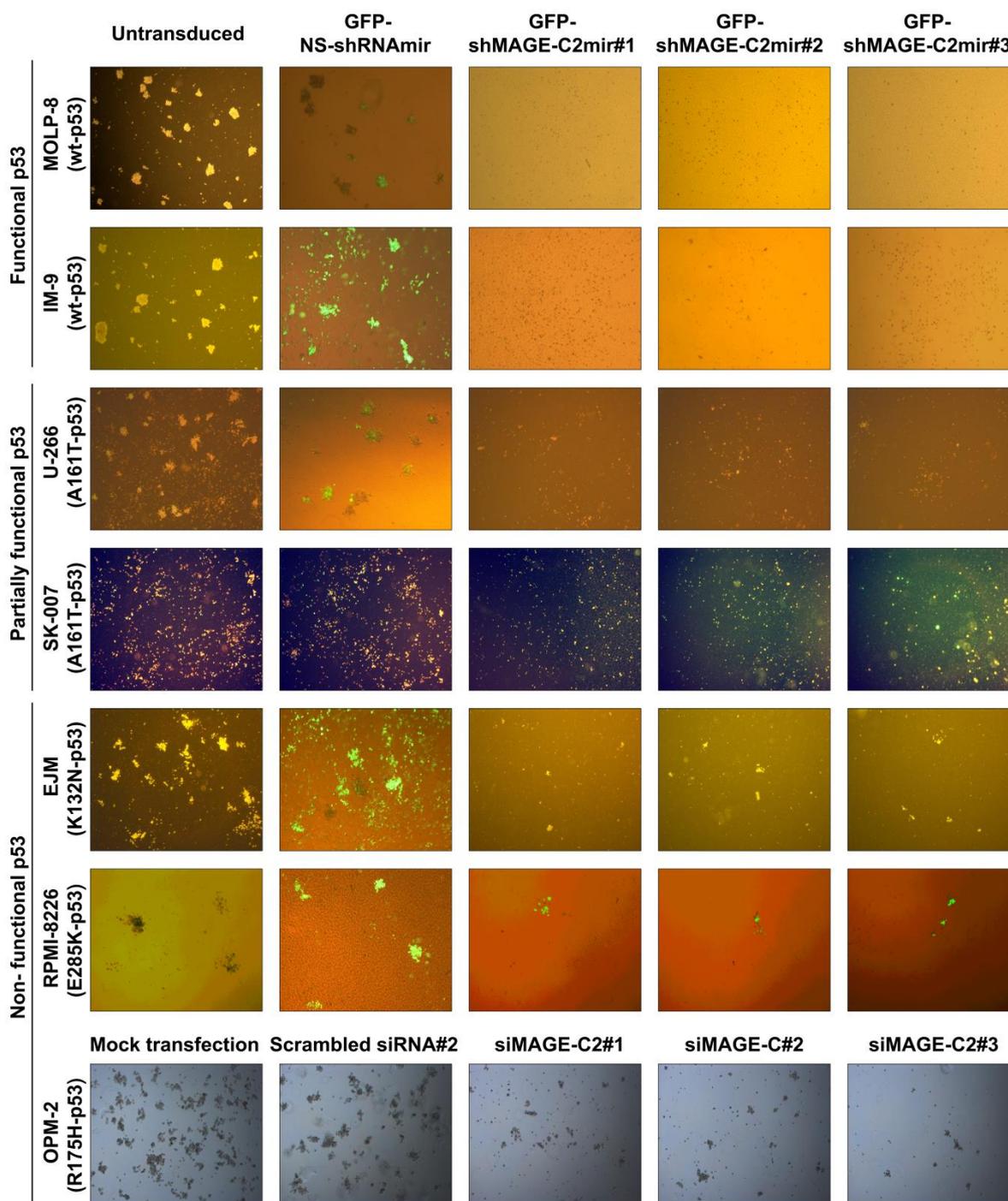


Figure 31. MAGE-C2/CT10 silencing inhibits the anchorage independent growth of myeloma cells. Colony formation assay of myeloma cell lines depleted or not from MAGE-C2 expression. Transiently transfected (OPM-2) and stably transduced myeloma cell lines were plated at 72h post-infection in Methylcellulose-Based Media and grown for 12 days. Snapshots were acquired when colonies are visible under phase inverted/ fluorescence microscope (Magnification x10).

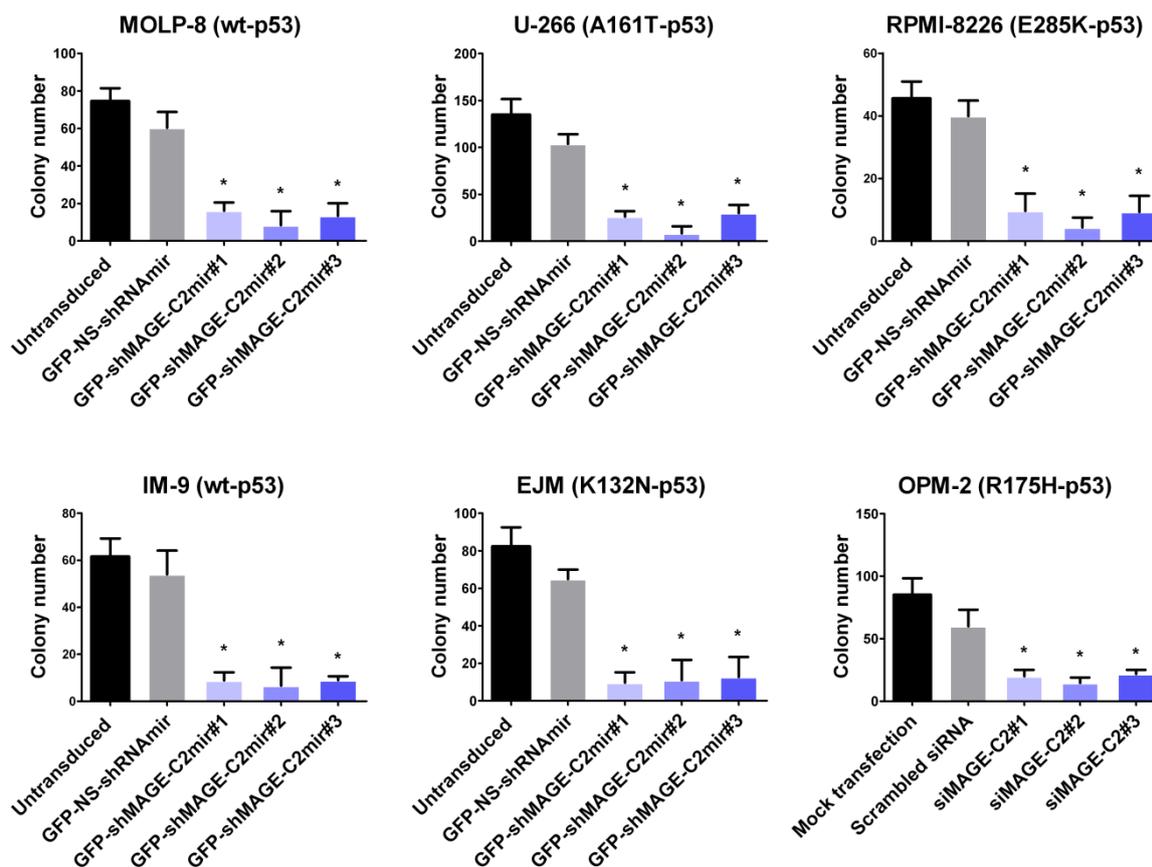


Figure 32. MAGE-C2/CT10 silencing inhibits the anchorage independent growth of myeloma cells. Histogram bars show the number of colonies consisting of at least 40 cells. The results present the mean of three independent experiments and error bars indicate standard deviation. Statistical significance was accepted with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing GFP-shMAGE-C2mir transduced cells to GFP-Non-Specific-shRNAmir transduced cells and siMAGE-C2 to scrambled siRNA#2.

VI. The analysis of the molecular mechanism of the anti-proliferative and pro-apoptotic effects of MAGE-C2/CT10 silencing

VI.1 MAGE-C2/CT10 silencing increases endogenous p53 level and induces the expression of cell cycle arrest p53 response elements

To understand the molecular basis of the anti-proliferative and pro-apoptotic effects of MAGE-C2 silencing on myeloma cell lines, we analysed the differential expression of 84 genes involved in the regulation of cell cycle progression in IM-9 (wt-p53) and U-266 (A161T-p53) expressing a functional and a mutated p53 that retains a partial transcriptional activity, respectively. In both myeloma cell lines, the heat-map (Figure 33, Figure 34, Table 7, Table 8) indicated that MAGE-C2 silencing induced more than two-fold increase in the expression levels of CDKN1A (p21^{WAF1/CIP1}), GADD45A (GADD45 α), CCNG1 (Cyclin G1) genes which are transcriptional targets of p53, known to control G1/S and G2/M cell cycle checkpoint and arrest in response to DNA damage [291-294]. However, no significant difference in the transcriptional level of p53, per se, was observed. Western blot analysis of whole cell lysates of MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53) and SK-007 (A161T-p53) depleted from MAGE-C2 expression showed, however, an accumulation of the endogenous level of p53 which is most likely due to protein stabilization and not an increase of p53 mRNA. In addition, we observed an increase in the endogenous level of p21^{WAF1/CIP1} which is the main p53 transcriptional target and inhibitor of cell cycle progression at G1/S and G2/M phases (Figure 39A), an effect which is absent from myeloma cells transduced with GFP-Non-specific-shRNAmir control vector suggesting that p53 stabilization and transactivation is due to the reduced level of MAGE-C2 expression.

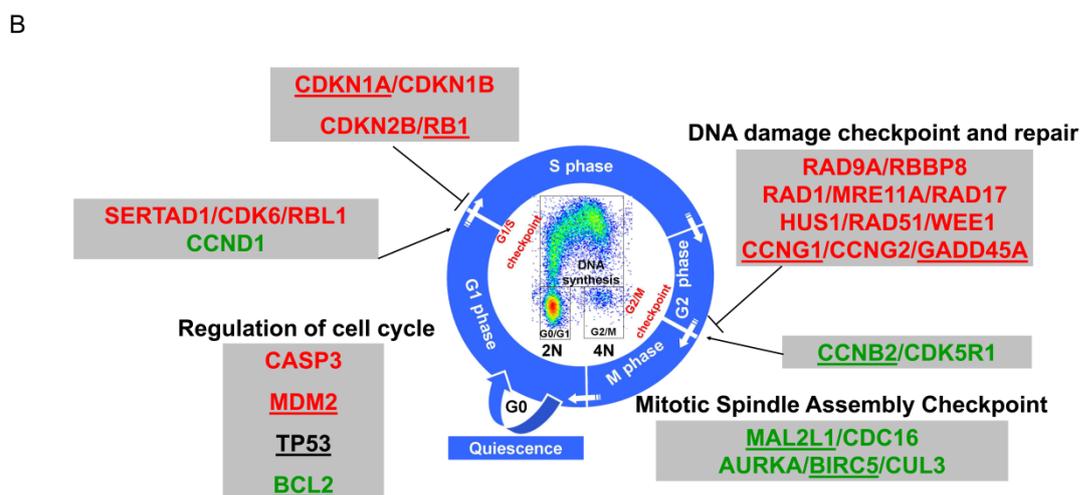
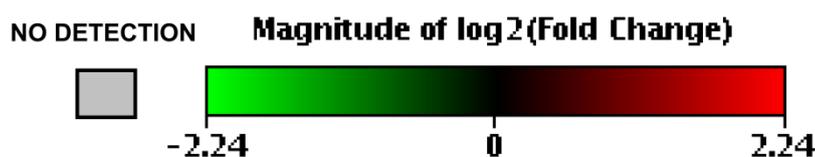
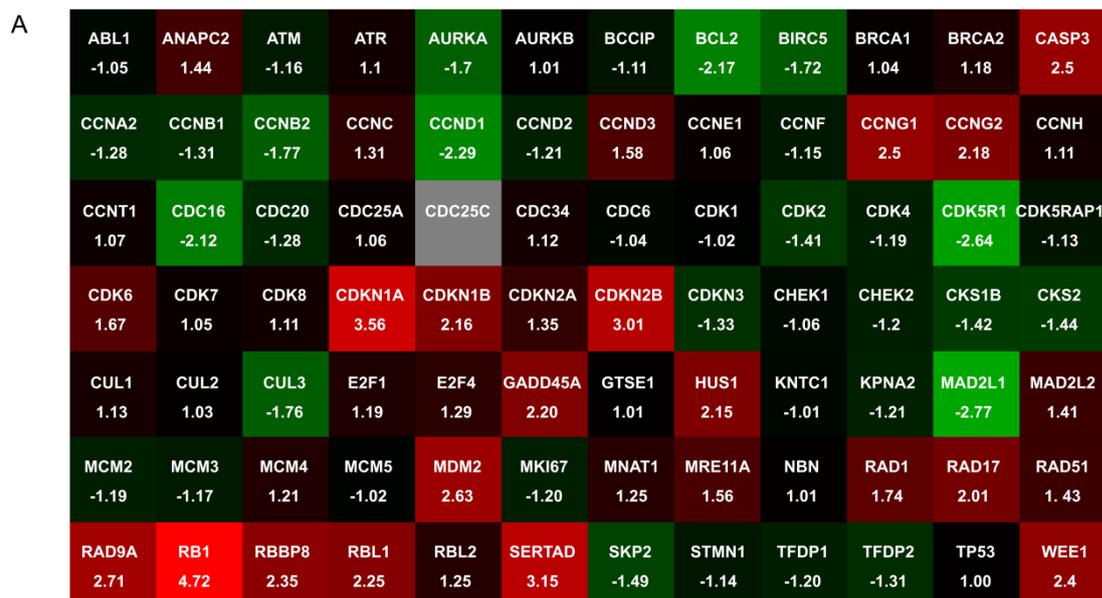


Figure 33. Differential expression of cell cycle related genes in IM-9 (wt-p53) depleted of MAGE-C2/CT10 expression. (A) The heat map presentation of expression profile of cell cycle related genes regulating negatively and positively cell cycle progression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). (B) Schematic presentation of the cell cycle and genes regulating positively (→) and negatively (—) cell cycle progression at different cell cycle phases and transitions that showed a differential regulation statistically significant ($P < 0.05$). Up-regulated genes are in red, down-regulated genes are in green and unchanged gene expression is shown in black. Underlined genes are transcriptionally regulated by p53.

Table 7. Differential expression of cell cycle related genes in IM-9 (wt-p53) depleted of MAGE-C2/CT10 expression.

Gene symbol, protein and cell cycle regulation *	Function*	Fold change	95% CI	P-value
G1 phase and G1/S transition				
CDK6 Cell division cycle 6 homolog (S. cerevisiae)	The activity of this kinase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase has been shown to phosphorylate, and regulate the activity of, tumor suppressor retinoblastoma protein.	1.67	(1.44, 1.90)	0.001
CCND1 Cyclin D1	This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. Mutations, amplification and overexpression of this gene, which alters cell cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis.	-2.29	(0.00001, 0.95)	0.3
G2 phase and G2/M transition				
CCNG1 Cyclin G1	The encoded protein is of the target genes of p53 and is involved in G2/M arrest in response to DNA damage.	2.5	(0.72, 4.29)	0.04
SERTAD1 SERTA domain containing 1	This protein stimulates E2F/DP-1 transcriptional activity and renders the activity of cyclin D1/CDK4 complex resistant to the inhibitory effect of p16 (INK4a).	3.15	(0.17, 6.13)	0.19
CDK5R1 Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	CDK5R1(p35)/ CDK5 is a key molecule in the cell cycle re-entry may stimulate cell survival (inhibit cell apoptosis) via Epidermal growth factor receptor family of receptor tyrosine kinases (ErbB).	-2.64	(0.30, 0.46)	0.003
CCNB2 Cyclin B2	Cyclin B2 forms with the CDC2 protein kinase the mitosis promoting factor complex which facilitates the progression from G2 to mitosis. The transcription of CCNB2 is repressed by p53.	-1.77	(0.29, 0.84)	0.14
M phase				
CDC25C Cell division cycle 25 homolog C (S. pombe)	The encoded protein is a tyrosine phosphatase and belongs to the Cdc25 phosphatase family. It directs dephosphorylation of cyclin B-bound CDC2 and triggers entry into mitosis. It is also thought to suppress p53-induced growth arrest.	49.29	(0.00001, 226.10)	0.04
CUL3 Cullin 3	Cul3 regulates mitosis and maintains the spindle Assembly checkpoint activity by ubiquitination of the chromosomal passenger protein Aurora B.	-1.7695	(0.40, 0.73)	0.042535

CDC16	CDC16 encodes a component protein of the APC complex is a cyclin degradation system that functions as a protein ubiquitin ligase and governs exit from mitosis.	-2.12	(0.06, 0.89)	0.263
BIRC5 Baculoviral IAP repeat containing 5	This gene is a member of the inhibitor of apoptosis (IAP) gene family, which encode negative regulatory proteins that prevent apoptotic cell death. IAP family members usually contain multiple baculovirus IAP repeat (BIR) domains, but this gene encodes proteins with only a single BIR domain. The encoded proteins also lack a C-terminus RING finger domain. Gene expression is high during fetal development and in most tumors, yet low in adult tissues.	-1.72	(0.27, 0.89)	0.09
MAD2L1 MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L1 is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate.	-2.77	(0.14, 0.58)	0.008
Cell cycle checkpoint and cell cycle arrest				
CCNG2 Cyclin G2	Cyclin G2 influences checkpoint signaling and is required for G2/M arrest in response to DNA damage. CCNG2 does not contain p53 binding sites and is a transcriptional target of the p53 homolog, p63.	2.18	(1.12, 3.26)	0.1
CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair.	3.56	(3.05, 4.07)	0.0004
CDKN1B Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.	2.16	(1.34, 3.00)	0.0273
CDKN2B Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression.	3.01	(2.10, 3.92)	0.003
GADD45A Growth arrest and DNA-damage-	This gene is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. The DNA damage-induced transcription of this gene is mediated by both p53-dependent and -independent	2.20	(1.29, 3.12)	0.03

inducible, alpha	mechanisms.			
HUS1 HUS1 checkpoint homolog (S. pombe)	The protein encoded by this gene is a component of an evolutionarily conserved, genotoxin-activated checkpoint complex that is involved in the cell cycle arrest in response to DNA damage. This protein forms a heterotrimeric complex with checkpoint proteins RAD9 and RAD1. In response to DNA damage, the trimeric complex interacts with another protein complex consisting of checkpoint protein RAD17 and four small subunits of the replication factor C (RFC), which loads the combined complex onto the chromatin. The DNA damage induced chromatin binding has been shown to depend on the activation of the checkpoint kinase ATM, and is thought to be an early checkpoint signaling event.	2.15	(0.25, 4.05)	0.11
MDM2	This gene is a target gene of the transcription factor tumor protein p53. The encoded protein is a nuclear phosphoprotein that binds and inhibits transactivation by tumor protein p53, as part of an autoregulatory negative feedback loop.	2.63	(1.50, 3.77)	0.004
MRE11A	This gene encodes a nuclear protein involved in homologous recombination, telomere length maintenance, and DNA double-strand break repair. By itself, the protein has 3' to 5' exonuclease activity and endonuclease activity. The protein forms a complex with the RAD50 homolog; this complex is required for nonhomologous joining of DNA ends and possesses increased single-stranded DNA endonuclease and 3' to 5' exonuclease activities.	1.56	(0.85, 2.28)	0.11
RAD1 RAD1 homolog (S. pombe)	This gene encodes a component of a heterotrimeric cell cycle checkpoint complex, known as the 9-1-1 complex, that is activated to stop cell cycle progression in response to DNA damage or incomplete DNA replication. The 9-1-1 complex is recruited by RAD17 to affected sites where it may attract specialized DNA polymerases and other DNA repair effectors.	1.74	(0.88, 2.61)	0.044
RAD17 RAD17 homolog (S. pombe)	A cell cycle checkpoint gene required for cell cycle arrest and DNA damage repair in response to DNA damage. This protein binds to chromatin prior to DNA damage and is phosphorylated by the checkpoint kinase ATR following damage. This protein recruits the RAD1-RAD9-HUS1 checkpoint protein complex onto chromatin after DNA damage, which may be required for its phosphorylation. The phosphorylation of this protein is required for the DNA-damage-induced cell cycle G2 arrest, and is thought to be a critical early event during checkpoint signaling in DNA-damaged cells.	2.01	(0.66, 3.38)	0.12
RAD9A RAD9 homolog A (S. pombe)	This gene product is highly similar to Schizosaccharomyces pombe rad9, a cell cycle checkpoint protein required for cell cycle arrest and DNA damage repair. This protein possesses 3' to 5' exonuclease activity, which may contribute to its role in sensing and repairing DNA damage. It forms a checkpoint protein complex with RAD1 and HUS1. This complex is recruited by checkpoint protein RAD17 to the sites of DNA damage, which is thought to be important for triggering the checkpoint-signaling cascade.	2.71	(0.77, 4.66)	0.08
RAD51	The protein encoded by this gene is known to be involved in the homologous recombination and repair of DNA. This protein is also	1.43	(1.11, 1.76)	0.03

RAD51 homolog (<i>S. cerevisiae</i>)	found to interact with BRCA1 and BRCA2, which may be important for the cellular response to DNA damage.			
RB1 Retinoblastoma 1	The protein encoded by this gene is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The encoded protein also stabilizes constitutive heterochromatin to maintain the overall chromatin structure. The active, hypophosphorylated form of the protein binds transcription factor E2F1. Defects in this gene are a cause of childhood cancer retinoblastoma (RB), bladder cancer, and osteogenic sarcoma.	4.72	(3.01, 6.44)	0.001
RBBP8 Retinoblastoma binding protein 8	The protein encoded by this gene is a ubiquitously expressed nuclear protein. It is found among several proteins that bind directly to retinoblastoma protein, which regulates cell proliferation. It is also associated with BRCA1 and is thought to modulate the functions of BRCA1 in transcriptional regulation, DNA repair, and/or cell cycle checkpoint control. Controls double strand break (DSB) resection, an event that occurs effectively only in S/G2 phases and that promotes homologous recombination but not non-homologous end joining.	2.35	(1.54, 3.16)	0.009
WEE1 WEE1 homolog (<i>S. pombe</i>)	This gene encodes a nuclear protein, which is a tyrosine kinase belonging to the Ser/Thr family of protein kinases. This protein catalyzes the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase, and appears to coordinate the transition between DNA replication and mitosis by protecting the nucleus from cytoplasmically activated CDC2 kinase.	2.40	(1.37, 3.44)	0.007
Regulation of cell cycle				
CASP3 Caspase 3, apoptosis-related cysteine peptidase	This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes which undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6, 7 and 9, and the protein itself is processed by caspases 8, 9 and 10.	2.52	(0.00001, 5.26)	0.26
TP53 Tumor protein p53	This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers, including hereditary cancers such as Li-Fraumeni syndrome.	1.009	(0.42, 1.60)	0.95
BCL2 B-cell CLL/lymphoma 2	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of BCL2, such as in the case of translocation of BCL2 to Ig heavy chain locus, is thought to be the cause of follicular lymphoma.	-2.17	(0.20, 0.72)	0.04

* Provided by the Reference sequence (RefSeq) data base

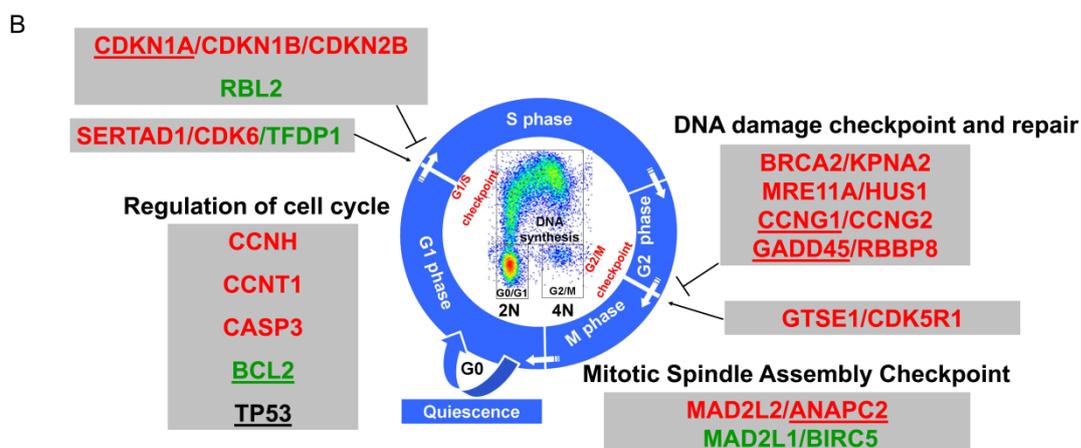
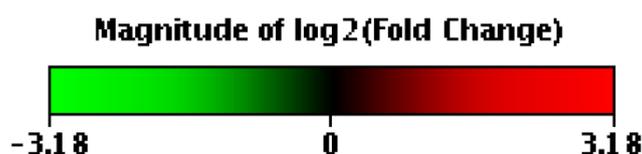
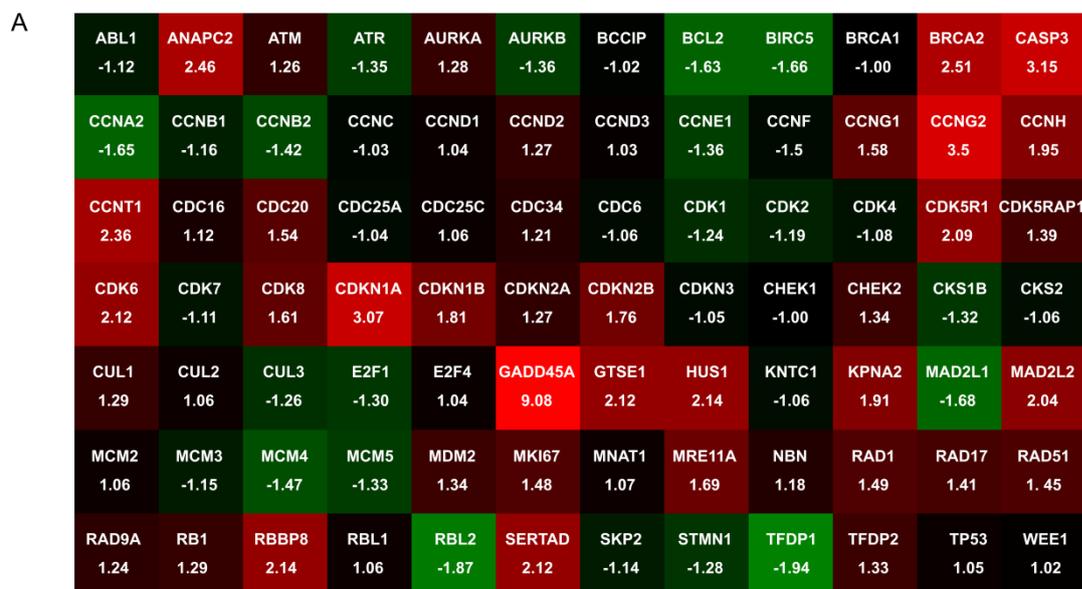


Figure 34. Differential expression of cell cycle related genes in U-266 (A161T-p53) depleted of MAGE-C2/CT10 expression. (A) The heat map presentation of expression profile of cell cycle related genes regulating negatively and positively cell cycle progression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). (B) Schematic presentation of the cell cycle and genes regulating positively (→) and negatively (—|) cell cycle progression at different cell cycle phases and transitions that showed a differential regulation statistically significant ($P < 0.05$). Up-regulated genes are in red, down-regulated genes are in green and unchanged gene expression is shown in black. Underlined genes are transcriptionally regulated by p53.

Table 8. Differential expression of cell cycle related genes in U-266 (A161T-p53) depleted of MAGE-C2/CT10 expression.

Gene symbol, protein and cell cycle regulation *	Function *	Fold change	95% CI	P-value
G1 phase and G1/S transition				
CDK6 Cell division cycle 6 homolog (S. cerevisiae)	The activity of this kinase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase has been shown to phosphorylate, and regulate the activity of, tumor suppressor retinoblastoma protein.	2.1289	(1.71, 2.54)	0.0001847
TFDP1 Transcription factor Dp-1	This gene encodes a member of a family of transcription factors that heterodimerize with E2F proteins to enhance their DNA-binding activity and promote transcription from E2F target genes. The encoded protein functions as part of this complex to control the transcriptional activity of numerous genes involved in cell cycle progression from G1 to S phase.	-1.9462	(0.30, 0.73)	0.063143
G2 phase and G2/M transition				
CDK5R1 Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	CDK5R1 (p35)/ CDK5 may stimulate cell survival (inhibit cell apoptosis) via Epidermal growth factor receptor family of receptor tyrosine kinases (ErbB).	2.0951	(0.88, 3.31)	0.048789
GTSE1 G-2 and S-phase expressed 1	The protein encoded by this gene is only expressed in the S and G2 phases of the cell cycle, where it colocalizes with cytoplasmic tubulin and microtubules. In response to DNA damage, the encoded protein accumulates in the nucleus and binds the tumor suppressor protein p53, shuttling it out of the nucleus and repressing its ability to induce apoptosis.	2.1293	(1.29, 2.96)	0.015837
SERTAD1 SERTA domain containing 1	This protein stimulates E2F/DP-1 transcriptional activity and renders the activity of cycline D1/CDK4 complex resistant to the inhibitory effect of p16(INK4a).	2.1244	(1.82, 2.43)	0.000684
M phase				
ANAPC2 Anaphase	A large protein complex, termed the anaphase-promoting complex (APC), or the cyclosome, promotes metaphase-anaphase transition by	2.4686	(1.77, 3.17)	0.007096

promoting complex subunit 2	ubiquitinating its specific substrates such as mitotic cyclins and anaphase inhibitor, which are subsequently degraded by the 26S proteasome.			
MAD2L2 MAD2 mitotic arrest deficient-like 2 (yeast)	The protein encoded by this gene is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate.	2.0425	(0.00001, 4.46)	0.191063
BIRC5 Baculoviral IAP repeat containing 5	This gene is a member of the inhibitor of apoptosis (IAP) gene family, which encode negative regulatory proteins that prevent apoptotic cell death. IAP family members usually contain multiple baculovirus IAP repeat (BIR) domains, but this gene encodes proteins with only a single BIR domain. The encoded proteins also lack a C-terminus RING finger domain. Gene expression is high during fetal development and in most tumors, yet low in adult tissues.	-1.6621	(0.38, 0.82)	0.059805
MAD2L1 MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L1 is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate.	-1.6814	(0.39, 0.80)	0.020417
Cell cycle checkpoint and cell cycle arrest				
BRCA2 Breast cancer 2, early onset	BRCA2 is a tumor suppressor gene that is involved in maintenance of genome stability, specifically the homologous recombination pathway for double-strand DNA repair. The BRCA2 protein contains several copies of a 70 amino acid motif called the BRC motif, and these motifs mediate binding to the RAD51 recombinase which functions in DNA repair.	2.5246	(1.97, 3.06)	0.004116
CCNG1 Cyclin G1	The encoded protein is of the target genes of p53 and is involved in G2/M arrest in response to DNA damage.	1.5878	(1.46, 1.71)	0.000686
CCNG2 Cyclin G2	CycG2 influences checkpoint signaling and is required for G2/M arrest in response to DNA damage. CCNG2 does not contain p53 binding sites, but recent work showed that CCNG2 is a transcriptional target of the p53 homolog, p63.	3.5073	(2.76, 4.26)	0.001127
CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this	3.0745	(2.71, 3.44)	0.000033

	gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair.			
CDKN1B Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.	1.8113	(1.05, 2.57)	0.051509
CDKN2B Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression.	1.7658	(1.15, 2.38)	0.0727
GADD45A Growth arrest and DNA-damage-inducible, alpha	This gene is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. The protein encoded by this gene responds to environmental stresses by mediating activation of the p38/JNK pathway via MTK1/MEKK4 kinase. The DNA damage-induced transcription of this gene is mediated by both p53-dependent and -independent mechanisms.	9.0653	(7.67, 10.46)	0.0001
HUS1 HUS1 checkpoint homolog (S. pombe)	The protein encoded by this gene is a component of an evolutionarily conserved, genotoxin-activated checkpoint complex that is involved in the cell cycle arrest in response to DNA damage. This protein forms a heterotrimeric complex with checkpoint proteins RAD9 and RAD1. In response to DNA damage, the trimeric complex interacts with another protein complex consisting of checkpoint protein RAD17 and four small subunits of the replication factor C (RFC), which loads the combined complex onto the chromatin. The DNA damage induced chromatin binding has been	2.149	(1.64, 2.66)	0.008781

	shown to depend on the activation of the checkpoint kinase ATM, and is thought to be an early checkpoint signaling event.			
KPNA2 Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2 regulates NBS1 (NBN), p27, Chk2 import to the nucleus.	1.919	(1.38, 2.46)	0.019187
MRE11A MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	This gene encodes a nuclear protein involved in homologous recombination, telomere length maintenance, and DNA double-strand break repair. By itself, the protein has 3' to 5' exonuclease activity and endonuclease activity. The protein forms a complex with the RAD50 homolog; this complex is required for nonhomologous joining of DNA ends.	1.6978	(1.53, 1.86)	0.000223
RBBP8 Retinoblastoma binding protein 8	The protein encoded by this gene is a ubiquitously expressed nuclear protein. It is found among several proteins that bind directly to retinoblastoma protein, which regulates cell proliferation. It is also associated with BRCA1 and is thought to modulate the functions of BRCA1 in transcriptional regulation, DNA repair, and/or cell cycle checkpoint control. Controls double strand break (DSB) resection, an event that occurs effectively only in S/G2 and that promotes homologous recombination but not non-homologous end joining.	2.1441	(1.83, 2.46)	0.000606
Regulation of cell cycle				
CASP3 Caspase 3, apoptosis-related cysteine peptidase	This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes which undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6, 7 and 9, and the protein itself is processed by caspases 8, 9 and 10.	3.1537	(2.38, 3.93)	0.005634
CCNH Cyclin H	CCNH plays a key role in cell cycle regulation by modulating the activity of cyclin-dependent kinase 7 (CDK7), which phosphorylates CDK1, 2, 4 and 6.	1.9548	(1.77, 2.14)	0.000505
CCNT1 Cyclin T1	Like cyclin H, the level of cyclin T does not oscillate during the cell cycle, suggesting that these cyclins	2.3626	(1.71, 3.01)	0.001868

	perform necessary functions that are not cell cycle stage specific.44-47 Cyclin T1 expression is regulated during T-cell activation and it is an important regulator of several cellular processes including lymphoid development. CCNT likely contributes to lymphomas derived from B- and T-cell lineages possibly through the inhibition of apoptosis.			
TP53 Tumor protein p53	This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers, including hereditary cancers such as Li-Fraumeni syndrome.	1.0548	(0.91, 1.20)	0.500823
BCL2 B-cell CLL/lymphoma 2	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of BCL2, such as in the case of translocation of BCL2 to Ig heavy chain locus, is thought to be the cause of follicular lymphoma.	-1.6392	(0.42, 0.80)	0.02519
RBL2 Retinoblastoma- like 2 (p130)	RBL2 regulates entry into the cell cycle.	-1.8786	(0.47, 0.59)	0.000835

* Provided by the Reference sequence (RefSeq) data base

Furthermore, IM-9 (wt-p53) and U-266 (A161T-p53) showed an up-regulation of a set of genes involved in DNA damage sensing, such as HUS1, MRE11A, RAD1, RAD9A, RAD17 [295] and in DNA damage repair such RAD51 which is known to be involved in the mitotic homologous recombination [296] indicating the presence of deleterious DNA. However, neither ataxia telangiectasia mutant (ATM) and Rad3-related protein kinase (ATR) which are the primary sensors of DNA lesions and the most upstream transducers of the DNA damage cascade [297], nor their immediate downstream effectors, the checkpoint kinase 1 (CHK1) and 2 (CHK2) [298] did show any significant differential expression following MAGE-C2 silencing.

Besides its trans-activation activity, p53 is also known to repress the expression of a number of genes including CCNB (Cyclin B1 and B2), CDK1 (CDC2) and CDC25C which facilitate the progression through G2/M transition [299, 300] as well as key components of the Mitotic

Spindle Assembly Checkpoint (SAC) that control accurate chromosome segregation during mitosis such as MAD2L1, BIRC5, AURKB, CDC20, CDC6 and STMN1. Indeed, we found that MAGE-C2 depletion and the consequent stabilization of p53 induced a significant down-regulation of MAD2L1 and BIRC5 expression in both cell lines (IM-9 (wt-p53), U-266 (A161T-p53)) and CCNB2, CDC16, CUL3 and AURKA expression in IM-9 (wt-p53). In tumor cells lacking functional p53, the overactivation of SAC due to an overexpression of the mitotic checkpoint genes is much more frequent than mitotic checkpoint loss or weakness which causes chromosomal instability and aneuploidy [301]. It could be that the down-regulation of the SAC components mediated by MAGE-C2 depletion may have normalized their levels and restored a functional mitotic checkpoint causing thus an inhibition of the progression through mitosis. These observations are in agreement with our findings demonstrating an increased percentage of G2/M population and an extended doubling time of myeloma cell lines depleted from MAGE-C2 expression. Taken together, these data provide functional evidences that MAGE-C2 silencing in myeloma cell lines, bearing functional p53 induces p53 accumulation and impairs cell cycle progression at G1/S and/or G2/M transitions in p53-dependent manner

VI.2 MAGE-C2/CT10 silencing activates the p53-dependent intrinsic apoptotic pathway

Besides p53-dependent repression of mitotic regulators, the cell cycle PCR arrays' results showed that MAGE-C2 depletion induced a suppression of BCL2 gene expression coupled to an increase of CASP3 gene expression, both of which represent two important compounds of the intrinsic apoptotic pathway. These findings prompted us to look at the differential expression of 84 genes encoding for pro- and anti-apoptotic proteins in order to identify the molecular mechanism that mediated cell death in myeloma cells depleted of MAGE-C2. Indeed, myeloma cell lines IM-9 (wt-p53) and U-266 (A161T-p53) increased the expression of TP73, GADD45A, TP53BP2, CIDEA, CIDEB, and ABL1 genes, which encode pro-apoptotic proteins mainly involved in the DNA damage response (Figure 35, Figure 37). We noted also an increase in the transcriptional levels of pro- apoptotic multi-BH domain Bcl2 genes, BAX and BAK1, which are the effectors of p53-dependent apoptosis and thought to be critical for DNA damage induced programmed cell death [302, 303]. Nonetheless, the activation of BAX and BAK depends on survival and death signals integrated by the BH3-only pro-proteins such as BIM (BCL2L11) , BAD, BID, PUMA and BIK. We found, indeed,

that MAGE-C2 silencing induced two-fold increase in BIK as well as in BID mRNA levels in U-266 (A161T-p53) which are positively regulated by p53 in order to assist the activation of BAX and BAK at the mitochondrial membrane [304, 305]. Moreover, myeloma cell lines depleted from MAGE-C2 expression up-regulated the expression of the initiator apoptotic caspase 9 and the apoptogenic protein APAF1 as well as the executioner apoptotic caspases 3, 6 (trans-activated by p53) and 7(Figure 36A, Figure 38A) [306].

Based on the fact that generally the fate of a cell depends on the ratio between pro-apoptotic BH3-only proteins and the pro-survival Bcl-2 proteins [307], we next looked at the differential expression of genes encoding for anti-apoptotic proteins. Both cell lines showed, indeed, a significant down-regulation of three anti-apoptotic genes known to be directly repressed by p53 namely of BCL2, MCL-1 and BIRC5 (Survivin). [308-310]. Furthermore, we found an up-regulation of other anti-apoptotic genes like AKT, NF- κ B and caspase inhibitors from BIRC family, likely counteracting the apoptotic signals induced by MAGE-C2 silencing. The differential expression of p53 transcriptional targets namely TP73, GADD45A, BAX, BAK, BID, caspase 6, APAF1, BCL2, BIRC5 and MCL-1 [311] indicates that MAGE-C2 silencing in myeloma cells activates p53-dependent-intrinsic apoptotic pathway which is dependent on mitochondria in response to cellular stress such as DNA damage or oncogenes activation (Figure 36B, Figure 38B) [312].

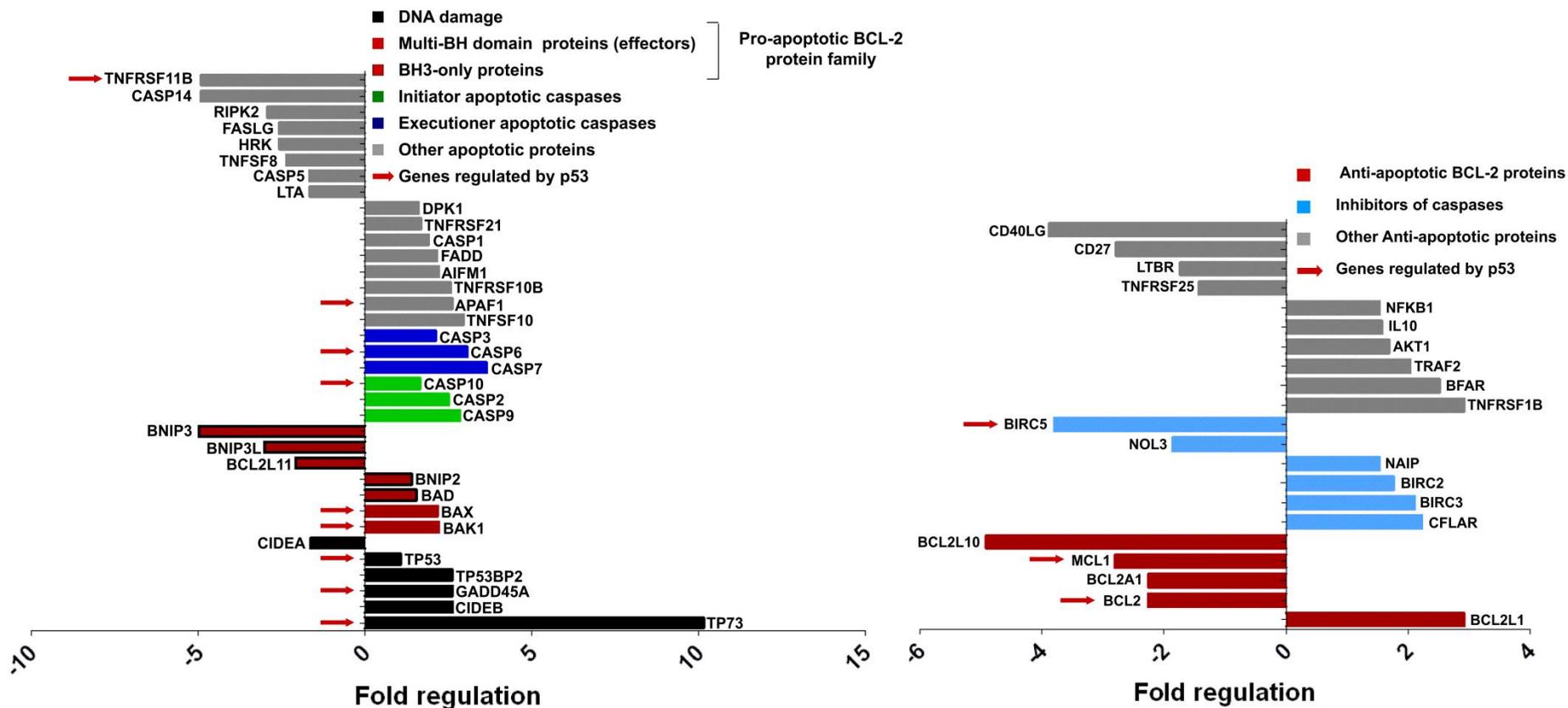


Figure 36. Differential expression of pro- and anti-apoptotic genes in IM-9 (wt-p53) depleted of MAGE-C2/CT10 expression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). (A) Bar graphs show fold regulation of pro-apoptotic genes (fold regulation > 1.5) that were grouped based on published data. (B) Bar graphs show fold regulation of anti-apoptotic proteins (fold regulation > 1.5) that were grouped based on published data. Red arrows point to genes regulated by p53.

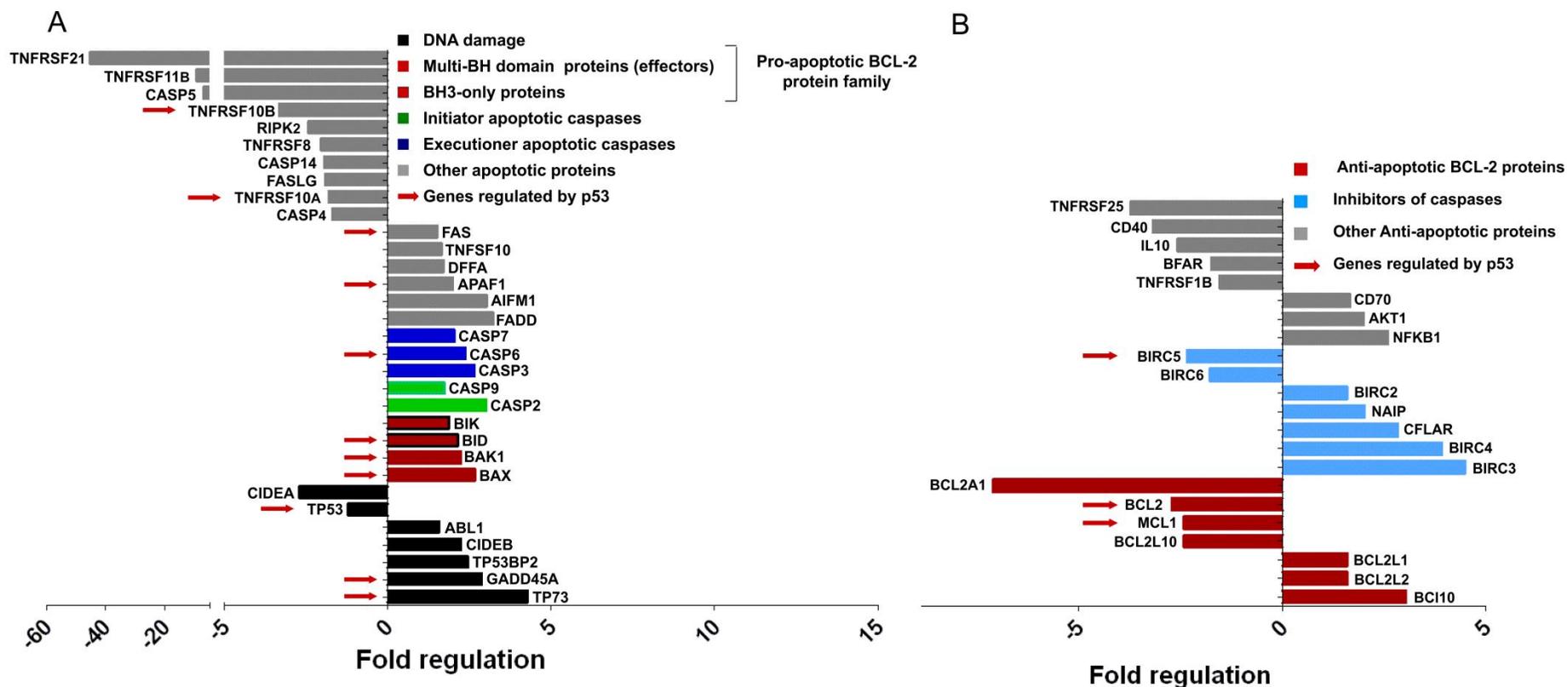


Figure 38. Differential expression of pro- and anti-apoptotic genes in U-266 (A161T-p53) depleted of MAGE-C2/CT10 expression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). **(A)** Bar graphs show fold regulation of pro-apoptotic genes (fold regulation > 1.5) that were grouped based on published data. **(B)** Bar graphs show fold regulation of anti-apoptotic proteins (fold regulation > 1.5) that were grouped based on published data. Red arrows point to genes regulated by p53.

To confirm our findings on the protein level, we next performed a western blot analysis of Bcl-2 pro-apoptotic protein expression in lysates of MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53) and SK-007 (A161T-p53) depleted of MAGE-C2 expression (Figure 39B). We found that the accumulation of p53 endogenous level was associated with an increase in BAX and BAK-1 protein expression. The analysis of BCL2L11 gene expression which encodes for 3 splicing variants of BH3-only protein, BIM: extra-long (Bim_{EL}) (the most predominant isoform), long (Bim_L) and short (Bim_s), revealed an increased level of the Bim's isoforms in U-266 (A161T-p53) and SK-007 (A161T-p53) associated to MAGE-C2 silencing but not in control cells. Among the three splicing variants, Bim_s, which is the most potent promoter of apoptosis and suppression of clonogenicity [313, 314], showed the highest increase in protein expression level following MAGE-C2 silencing. However, we found a very weak Bim expression in GFP-shMAGE-C2mir, GFP-Non-specific-shRNAmir transduced or untransduced IM-9 (wt-p53) and MOLP-8 (wt-p53) myeloma cell lines. A reduced Bim expression in myeloma cell lines had been previously reported to be due either to homozygote deletion or to the inhibitory effect of the insulin growth factor (IGF1). IGF1 is an important myeloma pro-survival factor that mediates its inhibitory effects by increasing BIM proteasomal mediated degradation, by decreasing BIM transcription through the activation of the AKT pathway or by silencing BIM promoter epigenetically [114].

In addition to the transcriptional regulation, the activity of Bcl-2 pro-apoptotic proteins is kept in check by post-translational modifications like phosphorylation. In MOLP-8 (wt-p53), MAGE-C2 silencing was associated with a dephosphorylation of BAD, which often occurs in response to death signal allowing its interaction with the anti-apoptotic proteins Bcl-xl and Bcl-2 and the neutralization of their pro-survival activity [315]. However, in IM-9 (wt-p53) and SK-007 (A161T-p53), we found an increase in BAD phosphorylation which is normally mediated by the pro-survival factor AKT [316]. Besides the post-translational modifications, some pro-apoptotic proteins might be activated by enzymatic cleavage such the case of pro-apoptotic protein BID (22kDa) which is cleaved by caspase 8 in response to TNF or Fas pathway activation, facilitating thus the cross talk between the extrinsic and intrinsic apoptotic pathways and amplification of the apoptotic signal [317]. Here, in the 4 cell lines we did not observe any cleavage of BID before or after MAGE-C2 silencing suggesting that there was no activation of the extrinsic pathway and the death signal was only due to the activation of the intrinsic apoptotic pathway in response to endogenous cellular stress. Altogether, these

findings show that MAGE-C2 silencing induces the activation of p53-dependent intrinsic pathway likely in response to irreversibly damaged DNA.

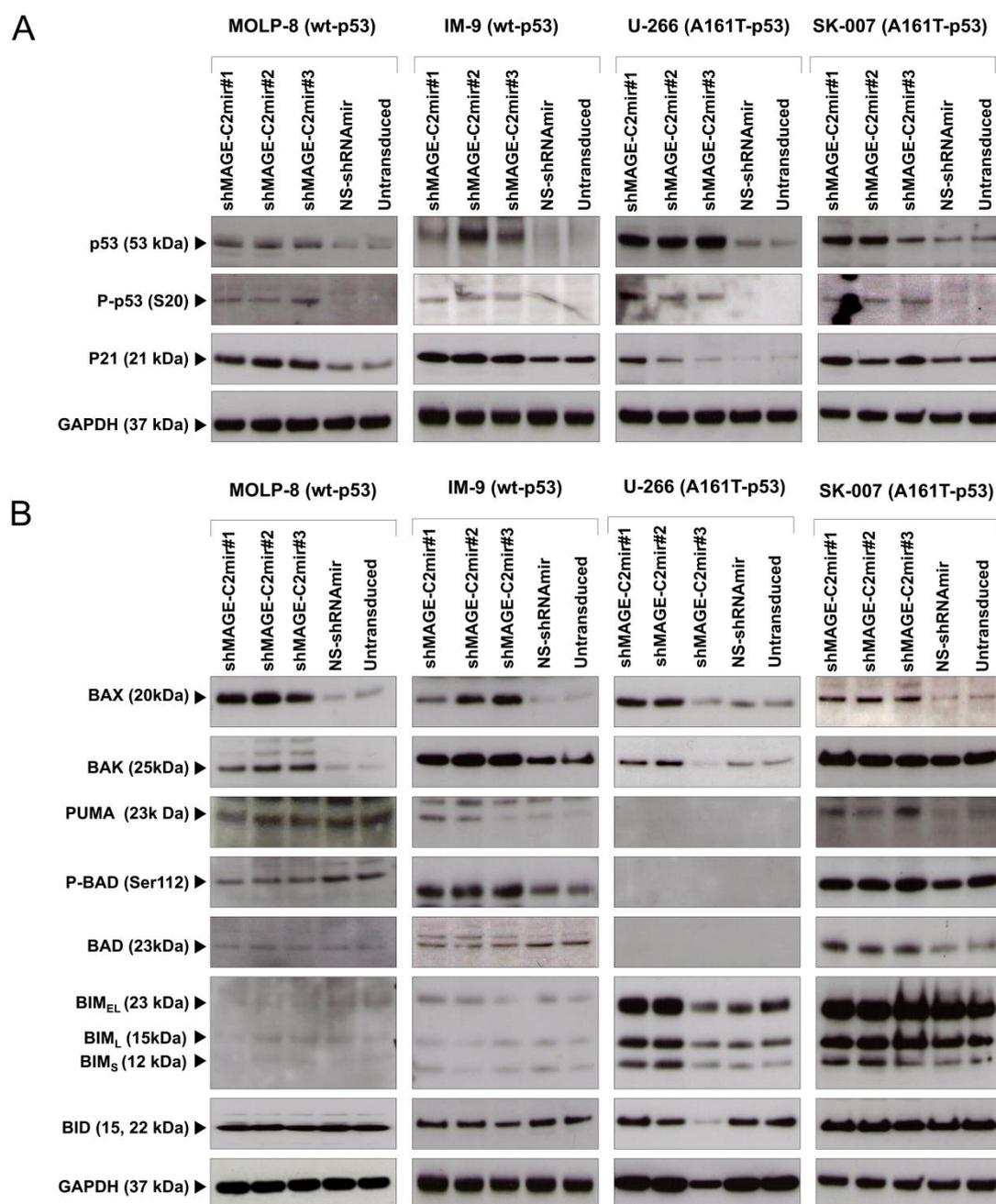


Figure 39. MAGE-C2/CT10 silencing increases p53 endogenous p53 level and induces the expression of cell cycle arrest and pro-apoptotic p53 response elements. (A) Western blot analysis of the p53 ((FL-393): sc-6243), phospho-p53 (S20) (#9287, Cell Signaling) and p21^(WAF1/CIP1) (#9287, Cell Signaling) expression in MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53), SK-007 (A161T-p53) Myeloma cell lines upon MAGE-C2/CT10 silencing. **(B)** Western blot analysis of Bcl-2 pro-apoptotic protein expression: BAX, BAK, PUMA, phospho-BAD (Ser112), BAD, BIM, BID in MOLP-8, IM-9, U-266, SK-007 Myeloma cell lines, upon MAGE-C2/CT10 silencing, using the Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit (#9942, Cell Signaling). GAPDH served as a loading control.

VI.3 MAGE-C2/CT10 silencing activates the E2F1-p73 pathway in the absence of functional p53

We next analysed the effect of MAGE-C2 depletion in myeloma cell line EJM, which expresses a rare p53 mutant K132N. K132N-p53 is a temperature sensitive mutant because of an altered tertiary structure at 37°C and above; impairing its bindings to the DNA sequence of p53 responsive elements and thus suppressing its transcriptional activity [318]. Intriguingly, despite the lack of functional p53 at standard culture condition, MAGE-C2 silencing induced a more than two-fold increase in CDKN1A and GADD45A mRNA expression as well as a significant decrease in the expression level of some components of the mitotic spindle checkpoint ANAPC2, MAD2L2, BIRC5, CDC16, CDC20, which may impair cell cycle progression and mitotic division (Figure 40, Table 9). We also noted a significant increase in the expression of a number of genes involved in DNA damage and repair responses (CHEK1, HUS1, RAD1, RAD51, BRCA1 and BRCA2) suggesting an activation of the DNA damage repair response, which may require a cell cycle delay or arrest.

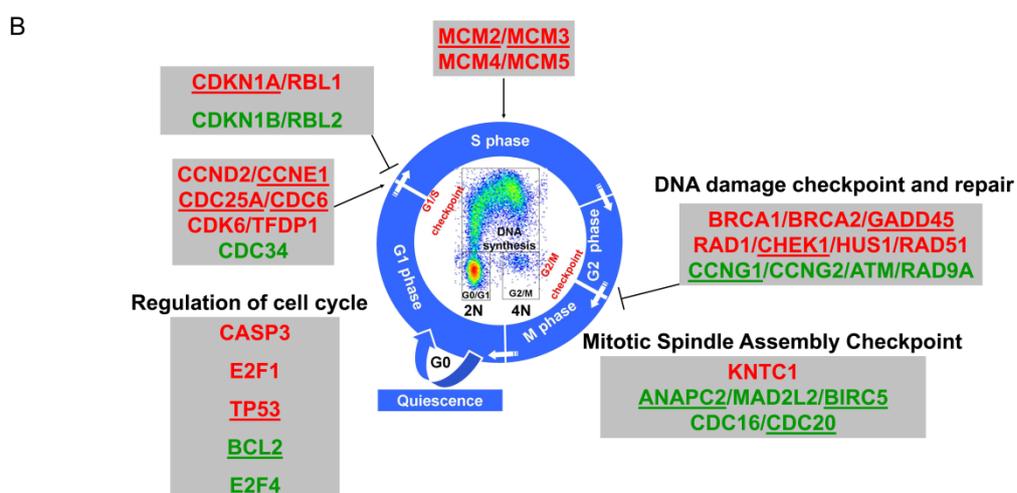
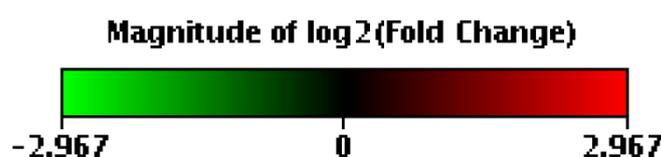
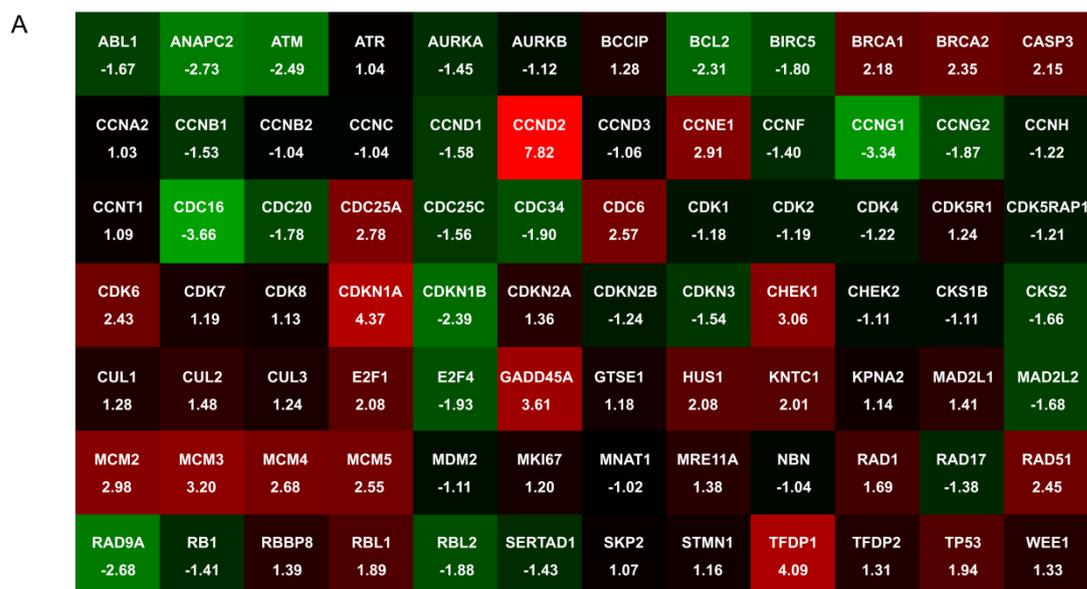


Figure 40. Differential expression of cell cycle related genes in EJM (K132N-p53) depleted of MAGE-C2/CT10 expression. (A) The heat map presentation of expression profile of 84 genes regulating negatively and positively cell cycle progression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). (B) Schematic presentation of the cell cycle and genes regulating positively (\longrightarrow) and negatively (\longleftarrow) cell cycle progression at different cell cycle phases and transitions that showed a differential regulation statistically significant ($P < 0.05$). Up-regulated genes are in red, down-regulated genes are in green and unchanged gene expression is shown in black. Underlined genes are transcriptionally regulated by p53.

Table 9. Differential expression of cell cycle related proteins in EJM (K132N-p53) depleted of MAGE-C2/CT10 expression

Gene symbol, protein and cell cycle regulation*	Function*	Fold change	95% CI	P-value
G1 phase and G1/S transition				
CCND2 cyclin D2	This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with and be involved in the phosphorylation of tumor suppressor protein Rb. Knockout studies of the homologous gene in mouse suggest the essential roles of this gene in ovarian granulosa and germ cell proliferation. High level expression of this gene was observed in ovarian and testicular tumors.	7.81	(6.04, 9.59)	0.001
CCNE1 cyclin E1	This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through S phase. Overexpression of this gene has been observed in many tumors, which results in chromosome instability, and thus may contribute to tumorigenesis. This protein was found to associate with, and be involved in, the phosphorylation of NPAT protein (nuclear protein mapped to the ATM locus), which participates in cell-cycle regulated histone gene expression and plays a critical role in promoting cell-cycle progression in the absence of pRB.	2.91	(2.15, 3.68)	0.005
CDC25A cell division cycle 25A	CDC25A is a member of the CDC25 family of phosphatases. CDC25A is required for progression from G1 to the S phase of the cell cycle. It activates the cyclin-dependent kinase CDC2 by removing two phosphate groups. CDC25A is specifically degraded in response to DNA damage, which prevents cells with chromosomal abnormalities from progressing through cell division. CDC25A is an oncogene, although its exact role in oncogenesis has not been demonstrated. Two transcript variants encoding different isoforms have been found for this gene.	2.77	(0.54, 5.01)	0.19
CDC6 cell division cycle 6	The protein encoded by this gene is highly similar to <i>Saccharomyces cerevisiae</i> Cdc6, a protein essential for the initiation of DNA replication. This protein functions as a regulator at the early steps of DNA replication. It localizes in cell nucleus during cell cycle G1, but translocates to the cytoplasm at the start of S phase. The subcellular translocation of this protein during cell cycle is regulated through its phosphorylation by Cdks. Transcription of this protein was reported to be regulated in response to mitogenic signals through transcriptional control mechanism involving E2F	2.57	(1.33, 3.82)	0.05

	proteins.			
CDK6 cyclin-dependent kinase 6	The protein encoded by this gene is a member of the cyclin-dependent protein kinase (CDK) family. CDK family members are highly similar to the gene products of <i>Saccharomyces cerevisiae cdc28</i> , and <i>Schizosaccharomyces pombe cdc2</i> , and are known to be important regulators of cell cycle progression. This kinase is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition. The activity of this kinase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase, as well as CDK4, has been shown to phosphorylate, and thus regulate the activity of, tumor suppressor protein Rb. Expression of this gene is up-regulated in some types of cancer. Multiple alternatively spliced variants, encoding the same protein, have been identified.	2.43	(0.92, 3.95)	0.08
RBL1 retinoblastoma- like 1 (p107)	The protein encoded by this gene is similar in sequence and possibly function to the product of the retinoblastoma 1 (RB1) gene. The RB1 gene product is a tumor suppressor protein that appears to be involved in cell cycle regulation, as it is phosphorylated in the S to M phase transition and is dephosphorylated in the G1 phase of the cell cycle. Both the RB1 protein and the product of this gene can form a complex with adenovirus E1A protein and SV40 large T-antigen, with the SV40 large T-antigen binding only to the unphosphorylated form of each protein. In addition, both proteins can inhibit the transcription of cell cycle genes containing E2F binding sites in their promoters. Due to the sequence and biochemical similarities with the RB1 protein, it is thought that the protein encoded by this gene may also be a tumor suppressor. Two transcript variants encoding different isoforms have been found for this gene.	1.88	(0.56, 3.21)	0.18
TFDP1 Transcription factor Dp-1	This gene encodes a member of a family of transcription factors that heterodimerize with E2F proteins to enhance their DNA-binding activity and promote transcription from E2F target genes. The encoded protein functions as part of this complex to control the transcriptional activity of numerous genes involved in cell cycle progression from G1 to S phase	4.09	(2.29, 5.90)	0.018
RBL2 retinoblastoma- like 2 (p130)		-1.87	(0.24, 0.82)	0.5
CDC34 cell division	The protein encoded by this gene is a member of the ubiquitin-conjugating enzyme family. Ubiquitin-conjugating enzyme catalyzes the covalent attachment of ubiquitin to other proteins. This protein is a part of the large multiprotein complex, which	-1.9	(0.34, 0.71)	0.01

cycle 34	is required for ubiquitin-mediated degradation of cell cycle G1 regulators, and for the initiation of DNA replication.			
S phase and DNA replication				
MCM2 minichromosome maintenance complex component 2	The protein encoded by this gene is one of the highly conserved mini-chromosome maintenance proteins (MCM) that are involved in the initiation of eukaryotic genome replication. The hexameric protein complex formed by MCM proteins is a key component of the pre-replication complex (pre_RC) and may be involved in the formation of replication forks and in the recruitment of other DNA replication related proteins. This protein forms a complex with MCM4, 6, and 7, and has been shown to regulate the helicase activity of the complex. This protein is phosphorylated, and thus regulated by, protein kinases CDC2 and CDC7. Multiple alternatively spliced transcript variants have been found, but the full-length nature of some variants has not been defined.	2.97	(0.78, 5.18)	0.15
MCM3 minichromosome maintenance complex component 3		3.20	(1.71, 4.69)	0.03
MCM4 minichromosome maintenance complex component 4		2.67	(0.08, 5.27)	0.22
MCM5 minichromosome maintenance complex component 5		2.54	(0.95, 4.15)	0.12
M phase				
ANAPC2 anaphase promoting complex subunit 2	A large protein complex, termed the anaphase-promoting complex (APC), or the cyclosome, promotes metaphase-anaphase transition by ubiquitinating its specific substrates such as mitotic cyclins and anaphase inhibitor, which are subsequently degraded by the 26S proteasome. Biochemical studies have shown that the vertebrate APC contains eight subunits. The composition of the APC is highly conserved in organisms from yeast to humans. The product of this gene is a component of the complex and shares sequence similarity with a recently identified family of proteins called cullins, which may also be involved in ubiquitin-mediated degradation.	-2.73	(0.19, 0.54)	0.02
CDC16 cell division cycle 16	CDC16 encodes a component protein of the APC complex is a cyclin degradation system that functions as a protein ubiquitin ligase and governs exit from mitosis.	-3.66	(0.05, 0.49)	0.01
CDC20	CDC20 appears to act as a regulatory protein interacting with several other proteins at multiple points in the cell cycle. It is	-1.78	(0.36, 0.77)	0.02

cell division cycle 20	required for two microtubule-dependent processes, nuclear movement prior to anaphase and chromosome separation.			
MAD2L2	The protein encoded by this gene is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. The encoded protein, which is similar to MAD2L1, is capable of interacting with ADAM9, ADAM15, REV1, and REV3 proteins.	-1.67	(0.40, 0.80)	0.02
BIRC5 Baculoviral IAP repeat containing 5	This gene is a member of the inhibitor of apoptosis (IAP) gene family, which encode negative regulatory proteins that prevent apoptotic cell death. IAP family members usually contain multiple baculovirus IAP repeat (BIR) domains, but this gene encodes proteins with only a single BIR domain. The encoded proteins also lack a C-terminus RING finger domain. Gene expression is high during fetal development and in most tumors, yet low in adult tissues.	-1.8	(0.37, 0.74)	0.02
Cell cycle checkpoint and cell cycle arrest				
BRCA1 Breast cancer 1, early onset	This gene encodes a nuclear phosphor-protein that plays a role in maintaining genomic stability, and it also acts as a tumor suppressor. The encoded protein combines with other tumor suppressors, DNA damage sensors, and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). This protein thus plays a role in transcription, DNA repair of double-stranded breaks, and recombination	2.17	(1.35, 3.01)	0.22
BRCA2 Breast cancer 2, early onset	BRCA2 is a tumor suppressor gene that is involved in maintenance of genome stability, specifically the homologous recombination pathway for double-strand DNA repair. The BRCA2 protein contains several copies of a 70 aa motif called the BRC motif, and these motifs mediate binding to the RAD51 recombinase which functions in DNA repair.	2.35	(1.63, 3.07)	0.017
RAD51 RAD51 homolog (S. cerevisiae)	The protein encoded by this gene is known to be involved in the homologous recombination and repair of DNA. This protein is also found to interact with BRCA1 and BRCA2, which may be important for the cellular response to DNA damage.	2.44	(1.19, 3.70)	0.08
CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. This protein was reported to be specifically cleaved by	4.36	(2.98, 5.76)	0.008

	CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation. Multiple alternatively spliced variants have been found for this gene.			
GADD45A Growth arrest and DNA-damage-inducible, alpha	This gene is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. The DNA damage-induced transcription of this gene is mediated by both p53-dependent and -independent mechanisms.	3.6	(0.65, 6.57)	0.12
HUS1 HUS1 checkpoint homolog (S. pombe)	The protein encoded by this gene is a component of an evolutionarily conserved, genotoxin-activated checkpoint complex that is involved in the cell cycle arrest in response to DNA damage. This protein forms a heterotrimeric complex with checkpoint proteins RAD9 and RAD1. In response to DNA damage, the trimeric complex interacts with another protein complex consisting of checkpoint protein RAD17 and four small subunits of the replication factor C (RFC), which loads the combined complex onto the chromatin. The DNA damage induced chromatin binding has been shown to depend on the activation of the checkpoint kinase ATM, and is thought to be an early checkpoint signaling event.	2.07	(0.90, 3.26)	0.06
KNTC1 kinetochore associated 1	This gene encodes a protein that is one of many involved in mechanisms to ensure proper chromosome segregation during cell division. Experimental evidence indicated that the encoded protein functioned in a similar manner to that of the Drosophila rough deal protein.	2.00	(0.41, 3.61)	0.17
RAD1 RAD1 homolog (S. pombe)	This gene encodes a component of a heterotrimeric cell cycle checkpoint complex, known as the 9-1-1 complex, that is activated to stop cell cycle progression in response to DNA damage or incomplete DNA replication. The 9-1-1 complex is recruited by RAD17 to affected sites where it may attract specialized DNA polymerases and other DNA repair effectors.	1.68	(1.26, 2.12)	0.01
CHEK1 checkpoint kinase 1	The protein encoded by this gene belongs to the Ser/Thr protein kinase family. It is required for checkpoint mediated cell cycle arrest in response to DNA damage or the presence of unreplicated DNA. This protein acts to integrate signals from ATM and ATR, two cell cycle proteins involved in DNA damage responses, that also associate with chromatin in meiotic prophase I. Phosphorylation of CDC25A protein phosphatase by this protein is required for cells to delay cell cycle progression in response to double-strand DNA breaks. Several alternatively spliced transcript variants have been found for this gene.	3.05	(1.27, 4.85)	0.066
CCNG1 Cyclin G1	The encoded protein is a target gene of p53 and is involved in G2/M arrest in response to DNA damage.	-3.34	(0.22, 0.38)	0.0001
CCNG2	Cycline G2 influences checkpoint signaling and is required for	-1.86	(0.08, 1.00)	0.27

Cyclin G2	G2/M arrest in response to DNA damage. CCNG2 does not contain p53 binding sites and is a transcriptional target of the p53 homolog, p63.			
CDKN1B Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.	-2.38	(0.18, 0.66)	0.015
ATM ataxia telangiectasia mutated	The protein encoded by this gene belongs to the PI3/PI4-kinase family. This protein is an important cell cycle checkpoint kinase that phosphorylates; thus, it functions as a regulator of a wide variety of downstream proteins, including tumor suppressor proteins p53 and BRCA1, checkpoint kinase CHK2, checkpoint proteins RAD17 and RAD9, and DNA repair protein NBS1. This protein and the closely related kinase ATR are thought to be master controllers of cell cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability.	-2.4911	(0.21, 0.60)	0.07
RAD9A RAD9 homolog A (S. pombe)	This gene product is highly similar to Schizosaccharomyces pombe rad9, a cell cycle checkpoint protein required for cell cycle arrest and DNA damage repair. This protein possesses 3' to 5' exonuclease activity, which may contribute to its role in sensing and repairing DNA damage. It forms a checkpoint protein complex with RAD1 and HUS1. This complex is recruited by checkpoint protein RAD17 to the sites of DNA damage, which is thought to be important for triggering the checkpoint-signaling cascade.	-2.67	(0.29, 0.46)	0.0001
Regulation of cell cycle				
CASP3 Caspase 3, apoptosis-related cysteine peptidase	This gene encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes which undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6, 7 and 9, and the protein itself is processed by caspases 8, 9 and 10.	2.15	(1.26, 3.05)	0.04
TP53 Tumor protein p53	This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers, including hereditary cancers	1.93	(0.78, 3.09)	0.04

	such as Li-Fraumeni syndrome.			
E2F1 E2F transcription factor 1	The protein encoded by this gene is a member of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses. The E2F proteins contain several evolutionally conserved domains found in most members of the family. These domains include a DNA binding domain, a dimerization domain which determines interaction with the differentiation regulated transcription factor proteins (DP), a transactivation domain enriched in acidic amino acids, and a tumor suppressor protein association domain which is embedded within the transactivation domain. This protein and another 2 members, E2F2 and E2F3, have an additional cyclin binding domain. This protein binds preferentially to retinoblastoma protein pRB in a cell-cycle dependent manner. It can mediate both cell proliferation and p53-dependent/independent apoptosis.	2.07	(0.04, 4.12)	0.3
E2F4 E2F transcription factor 4, p107/p130-binding	The protein encoded by this gene is a member of the E2F family of transcription factors. This protein binds to all three of the tumor suppressor proteins pRB, p107 and p130, but with higher affinity to the last two. It plays an important role in the suppression of proliferation-associated genes, and its gene mutation and increased expression may be associated with human cancer.	-1.93	(0.27, 0.77)	0.05
BCL2 B-cell CLL/lymphoma 2	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of BCL2, such as in the case of translocation of BCL2 to Ig heavy chain locus, is thought to be the cause of follicular lymphoma	-2.31	(0.34, 0.52)	0.0004

* Provided by the Reference sequence (RefSeq) data base

Furthermore, the analysis of the differential expression of the pro- and anti-apoptotic genes showed that similarly to IM-9 (wt-p53) and U-266 (A161T-p53), MAGE-C2 depletion in EJM (K132N-p53) induced an up-regulation of the pro-apoptotic genes involved in the DNA damage response such as TP73, GADD45A, CIDEA and CIDEB, more than two-fold increase in the mRNA level of components of the intrinsic apoptotic pathway namely BAX, BAK1, the initiator apoptotic caspase 9 and the executioner apoptotic caspase 3 and 7 (Figure 41, Figure 42). These findings demonstrate that even in the absence of functional p53, MAGE-C2 depletion may impair cell cycle progression and activate the intrinsic apoptotic pathway in p53 similar manner.

Because of shared sequence homology and functional similarities with p53, the tumor suppressor p73 can induce G1/S and G2/M arrest in p53 similar manner by activating the

expression of some p53 gene targets such as CDKNA1, GADD45A, and TNFRSF10A (DR5) by repressing CCNB1. Moreover, p73 may play a role in E2F1 induced apoptosis by up-regulating BAX and PUMA expression [319-325]. Like p53, E2F1 is also a transcription factor and an important regulator of apoptosis in transformed or DNA-damaged cells and it is involved in both p53-dependent and independent apoptosis in response to DNA damage. E2F1 regulates the expression of a number of genes some of which are in common with p53, such as TP73, BAK1, BAD, BID, APAF1, CASP (3, 7, 8, 9), TP53 per se and the repression of the anti-apoptotic genes BCL-2 and MCL1 [326]. We, therefore, believe that E2F1-p73 pathway might be responsible for the cycle arrest and apoptosis associated to MAGE-C2 silencing in EJM (E285K-p53) lacking functional p53.

Interestingly, in contrast to IM-9 (wt-p53) and U-266 (A161T-p53), which showed unchanged or reduced p53 mRNA level following MAGE-C2 knockdown, two-fold increase in p53 transcriptional level was detected in both cell cycle and apoptosis arrays of EJM (K132N-p53) myeloma cell line depleted of MAGE-C2 expression which might be due to E2F1 transcriptional activity. We also noted a significant increase in the mRNA expression level of the pro-apoptotic genes APAF1, BAK1, CASP3, CASP7, CASP9 and TP73 as well as a significant decrease in the RNA level of the anti-apoptotic genes BCL2 and MCL1 (Figure 42) which might be attributable to E2F1 activation.

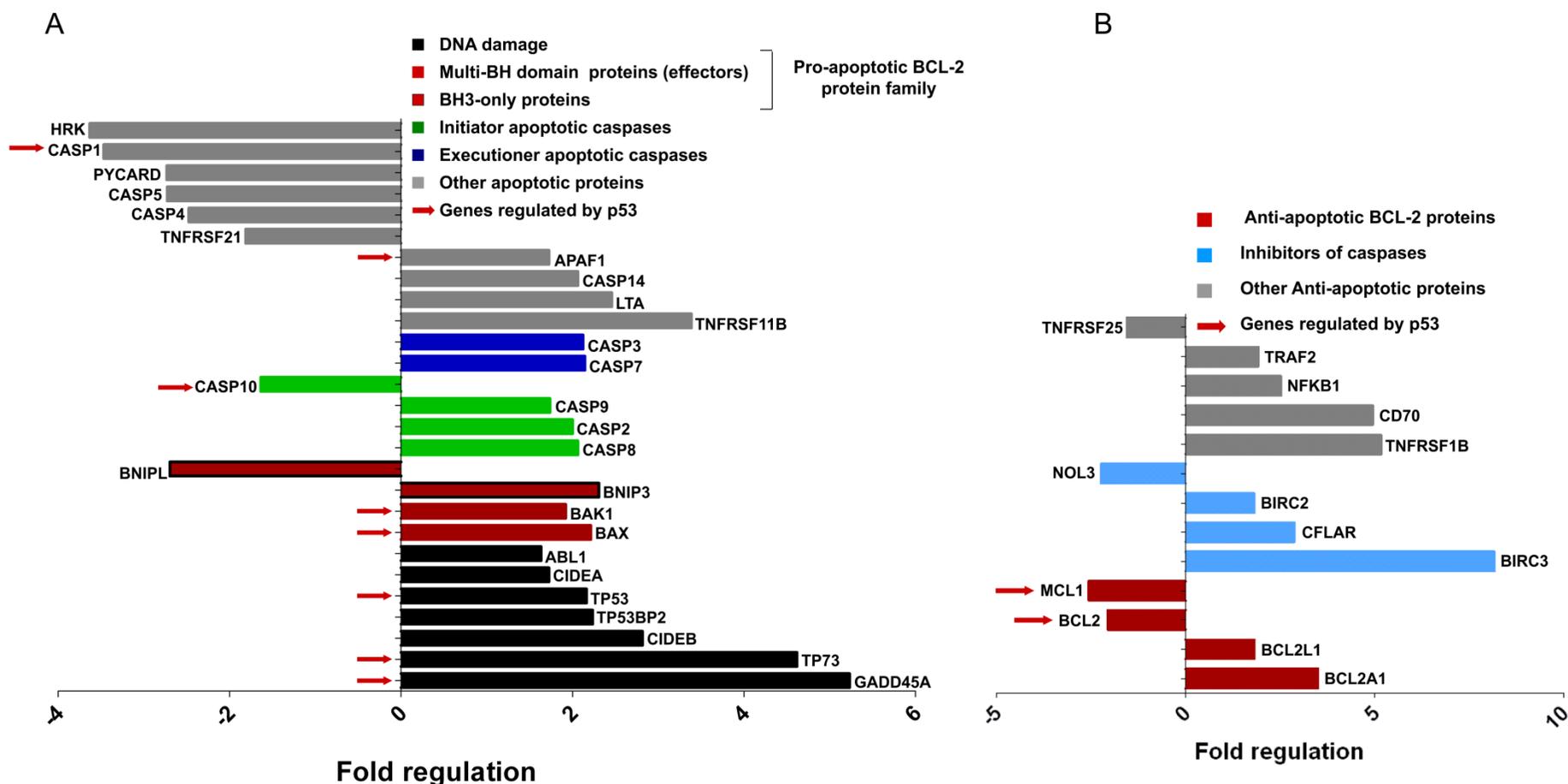
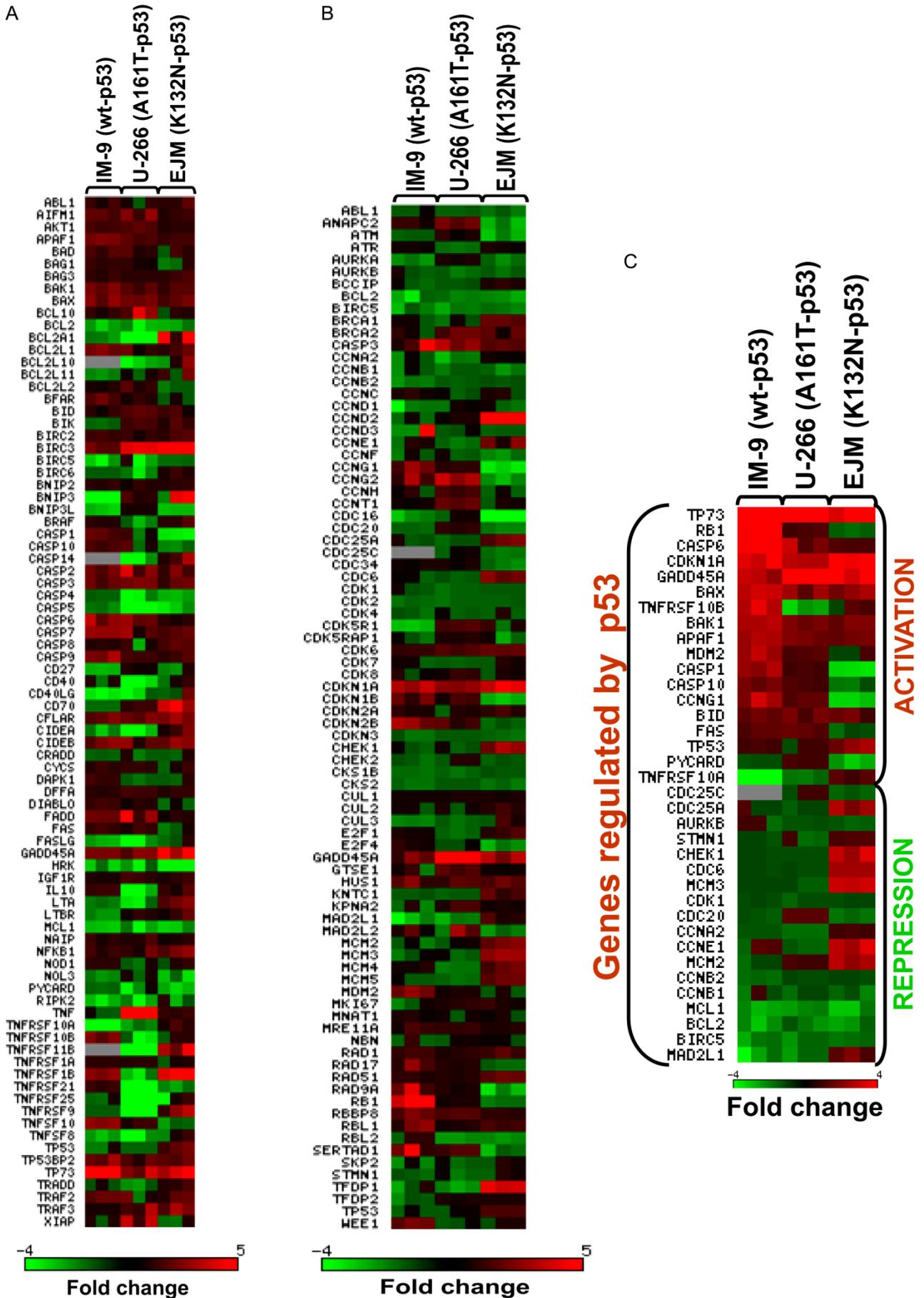
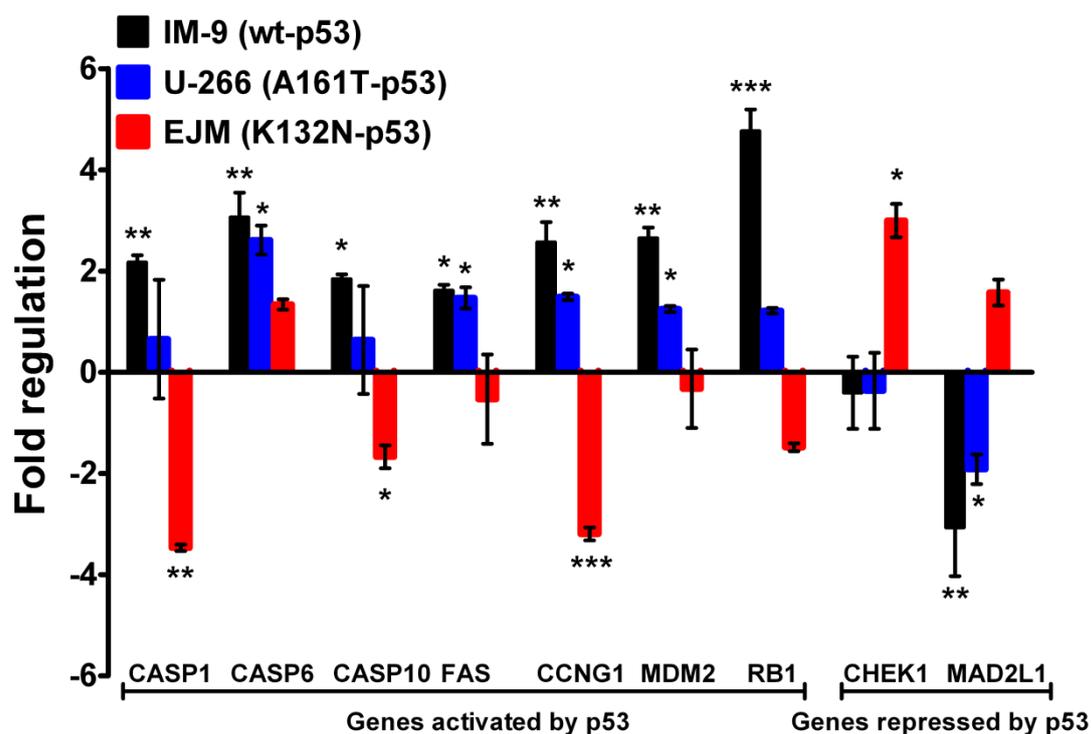


Figure 42. Differential expression of pro- and anti-apoptotic genes in EJM (K132N-p53) depleted of MAGE-C2/CT10 expression. Data are representative of the fold regulation (GFP-shMAGE-C2mir versus GFP-NS-shRNAmir) of three independent experiments (Fold regulation positive= up-regulation, Fold regulation negative=down-regulation). **(A)** Bar graphs show fold regulation of pro-apoptotic genes (fold regulation > 1.5) that were grouped based on published data. **(B)** Bar graphs show fold regulation of anti-apoptotic proteins (fold regulation > 1.5) that were grouped based on published data. Red arrows point to genes regulated by p53.

To discard the possibility that K132N-p53 mutant may retain a partial transcriptional activity at 37C°, we defined a group of genes directly activated or repressed by p53 and their differential expression in IM-9 (wt-p53), U-266 (A161T-p53) and EJM (K132N-p53) myeloma cell lines depleted of MAGE-C2 expression, was compared by heat map analysis (Figure 43A, B, C). While, U-266 (A161T-p53) represented an intermediate p53 gene expression profile, consistent with the partial loss of the transcriptional activity associated to A161T mutation, EJM (K132N-p53) showed an impaired activation and repression of a number of genes. The comparison of the differential expression of genes exclusively regulated by p53 in EJM (K132N-p53), U-266 (A161T-p53) and IM-9 (wt-p53), showed that EJM (K132N-p53) myeloma cells depleted from MAGE-C2 down-regulated genes normally activated by p53 (CASP6, CASP10, FAS, RB1, MDM2, CCNG1) and up-regulated genes normally repressed by p53 (MAD2L1 and CHEK1) (Figure 43D). Conversely, the analysis of the E2F1 target genes differential expression demonstrated an increased expression in EJM (K132N-p53), while such effect was not observed in U-266 (A161T-p53) and IM-9 (wt-p53) (Figure 43E). Altogether, our results indicate that, in the absence of functional p53, MAGE-C2 silencing may induce cell cycle arrest and apoptosis of myeloma cells, in p53 similar manner, likely, through the activation of E2F1-TP73 apoptotic pathway in response to DNA damage.



D Differential regulation of p53 target genes



E Differential regulation of E2F target genes

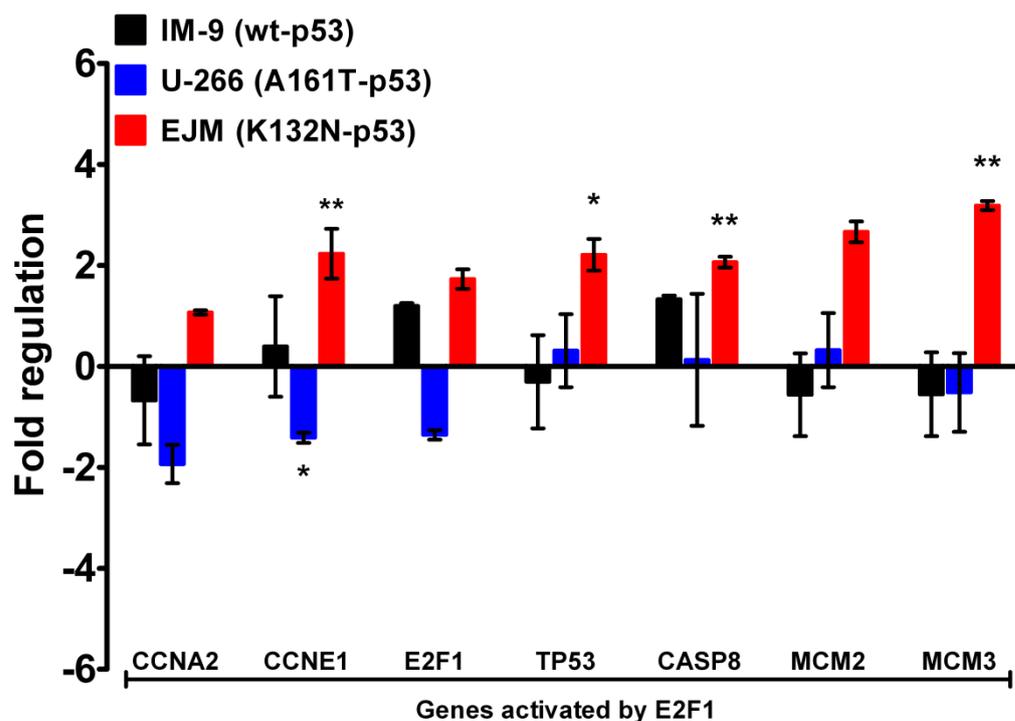


Figure 43. MAGE-C2/CT10 silencing increases the transcriptional activity of E2F1 in the absence of functional p53. (A) The heat map presentation of cell cycle and (B) apoptosis array showing the differential expression of 168 genes in IM-9 (wt-p53), U-266 (A161T-p53) and EJM (K132N-p53). Rows are genes, columns are cell lines. Each cell line is presented by 3 columns corresponding to differential gene expression of three independent experiments. The heat map was created with matrix2png (Pavlidis, P. and Noble W.S. *Bioinformatics*, 2003) and the values plotted are log ratio of fold change. (C) The heat map presentation of the differential expression of genes activated and repressed by p53 in IM-9 (wt-p53), U-266 (A161T-p53) and EJM (K132N-p53) upon MAGE-C2 silencing. Rows are genes, columns are cell lines. Each cell line is represented by 3 columns corresponding to differential gene expression in three independent experiments. The heat map was created with matrix2png and the values plotted are log ratio of fold change. (D) Bar graph shows the differential regulation of p53 target genes. (E) Bar graph shows the differential regulation of E2F1 target genes.

The analysis of protein expression, in both cell lines EJM (K132N-p53) and RPMI-8226 (E285K-p53) expressing non-functional p53, showed that MAGE-C2 knockdown induced an accumulation of p53 endogenous level probably due to an enhanced transcription (Figure 44). Using the well-established anti-p73 polyclonal antibody, H79 [327, 328], we found that MAGE-C2 knockdown up-regulated two major p73 isoforms, proapoptotic TAp73 α and TAp73 β which are induced by E2F1, differentially transactivate p53 responsive promoters (CDKN1 and BAX) and induce cell cycle arrest and apoptosis in response to DNA damage [325, 329, 330]. Accordingly, MAGE-C2 depletion increased p21^{WAF1/CIP1} and BAX protein expression levels. Despite E2F1 mRNA up-regulation in EJM (K132N-p53), there was no difference in E2F1 protein expression between controls or MAGE-C2 depleted cells. Nonetheless, we detected an increase in E2F1 target gene products namely BAK1, BAD, BIM and PUMA pro-apoptotic proteins. These findings suggest that unlike MAGE-C2/KAP1 mediated p53 degradation; MAGE-C2 may alter the pro-apoptotic activity of E2F1 likely by suppressing its transcriptional activity. Nonetheless, apoptosis and DNA repair are not the only biological processes regulated by E2F1, it has been shown that E2F1 may also promote DNA replication and proliferation and the tight regulation of its expression and /or stability dictated by the cellular context may shift the balance between the proliferative and apoptotic signals [331]. However, if we assume that K132N-p53 mutant may keep a residual activity, p53 and E2F1-p73 apoptotic pathways may promote cooperatively the apoptotic cell death in EJM (K132N-p53) and RPMI-8226 (E285K-p53) myeloma cells depleted of MAGE-C2 expression.

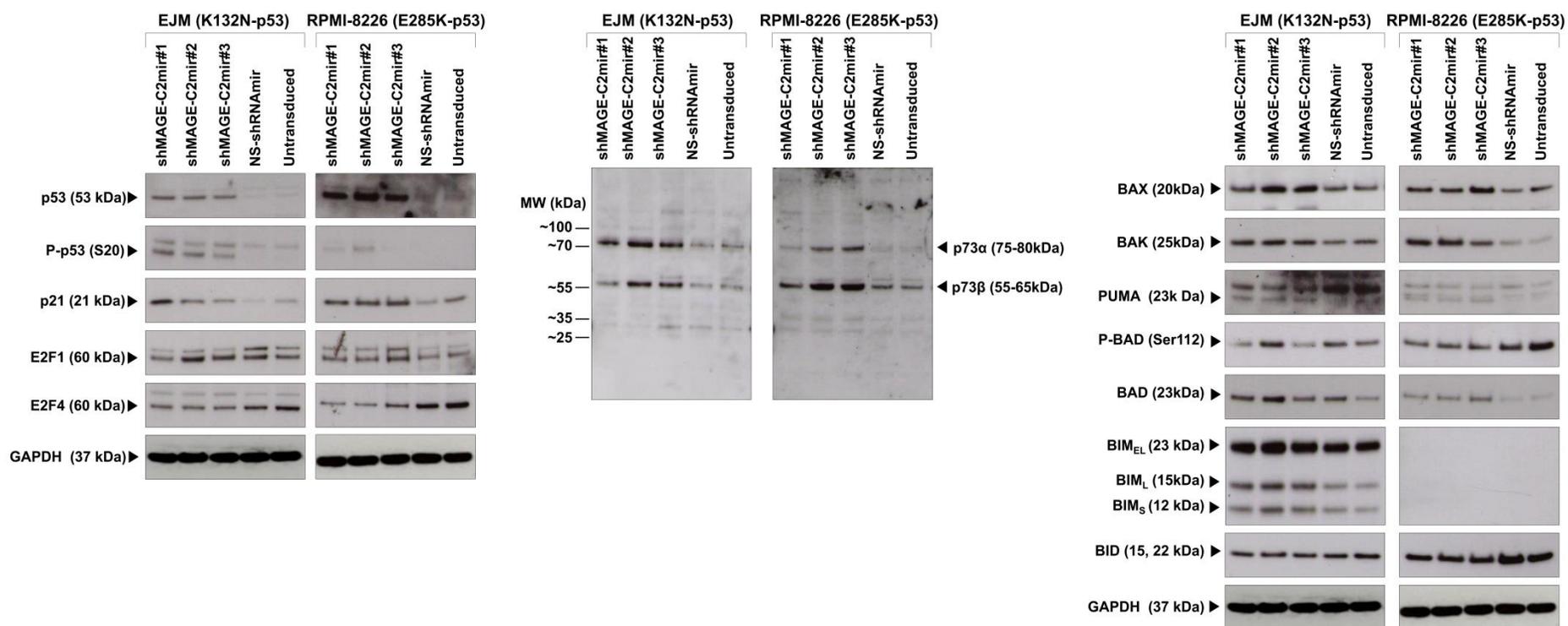


Figure 44. MAGE-C2/CT10 silencing activates E2F1-p73 apoptotic pathway in the absence of functional p53. Western blot analysis of p53, phospho-p53 (Ser20), p21^(WAF1/CIP1), E2F1 ((C-20):sc-193), E2F4 ((C-20):sc-866), TAp73 α , TAp73 β ((H-79):sc-7957) and Bcl-2 pro-apoptotic protein expression: BAX, BAK, PUMA, phospho-BAD (Ser112), BAD, BIM, BID in EJM and RPMI-8226 Myeloma cell lines, upon MAGE-C2 silencing. GAPDH served as a loading control.

VI.4 MAGE-C2/CT10 silencing enhances the ATM/ATR-CHK1/CHK2 DNA damage response

The accumulation of p53 endogenous level, by reduced protein degradation, is not sufficient to integrate genotoxic or oncogenic stress into a biological outcome. In addition, accumulated p53 needs to undergo some post-translational modification activating its transcriptional activity which will result in cell cycle arrest, DNA repair, apoptosis or senescence. The acetylation of p53, which was described to be suppressed by MAGE class I family members, enhances sequence-specific DNA binding and activates p53-mediated transactivation of its down-stream pro-apoptotic target genes. However, in response to DNA damage, the acetylation can be interdependent of p53 phosphorylation, required as a first post-translational modification for the recruitment of acetyl-transferases [332]. These observations prompted us to examine the phosphorylation status of p53 in MOLP-8 (wt-p53), IM-9 (wt-p53), U-266 (A161T-p53) and SK-007 (A161T-p53) using anti-p53 antibodies that recognize phosphorylated epitopes. Western blot analysis showed that MAGE-C2 silencing induced a phosphorylation of p53 on the serine 20 residue which is a post-translational modification known to activate p53 transcriptional activity in response to DNA damage [333] (Figure 39A). We also found that p53 mutants in EJM and RPMI-8226 cell lines to be phosphorylated at Ser20 following MAGE-C2 silencing (Figure 44). However, this event might be effective in stabilizing p53 but not in enhancing p53 transactivation activity since these conformational mutants are unable of DNA binding. The phosphorylation of p53 on serine 20 is mediated by activated serine/ threonine checkpoint kinase 1 (CHK1) and 2 (CHK2), the main effectors of the DNA damage signaling pathway coordinating G1/S, G2/M cell cycle arrest and DNA repair [217, 334]. On the basis of these observations, we analyzed the activation status of CHK1 and CHK2 as well as other key compounds of the DNA damage-signaling pathway. Western blot analysis revealed, in all myeloma cell lines, a constitutive phosphorylation of CHK2 and/or CHK1 at Ser296 and Thr68, respectively, that was enhanced following MAGE-C2 silencing (Figure 45B). In MOLP-8, SK-007, EJM and RPMI-8226, there was an increase of the inhibitory phosphorylation of Cdc2 (CDK1), a cyclin-dependent kinase essential to mitotic entry and exit, which might be responsible for the pronounced delay at G2/M transition shown by the cell cycle kinetic analysis. The phosphorylation and activation of CHK1 and CHK2 are selectively mediated by ATM and ATR which are important upstream regulators of p53 and required for G1/S and G2/M cell cycle arrest and DNA repair [335-339].

Western blot analysis demonstrated a constitutive phosphorylation of ATM and ATR, at Ser1981 and Ser428, respectively, suggesting the presence of ongoing DNA damage in myeloma cell lines (Figure 45A). A sensitive indicator of the DNA damage is the phosphorylation of the histone variant H2A.X on Ser139 which is a hallmark of the double strand breaks (DSB), a deleterious lesions that contribute to genomic instabilities. The phosphorylation of H2A.X is mediated by ATM/ATR to provide a signal for the recruitment of proteins involved in chromatin remodeling and DNA repair to the site of DSB. Our analysis, indeed, showed, a constitutive phosphorylation of the histone variant H2AX at Ser139 which further increased with MAGE-C2 silencing (Figure 45A). The investigation of the phosphorylation status of BRCA1 which is an important compound of DNA repair machinery did not reveal any phosphorylation in all myeloma cell lines (data not shown). We noted only an increase in the endogenous level of BRCA1 in SK-007, RPMI-8226 and EJM, while MOLP-8 and U-266 were negative for BRCA1 protein expression. Despite an increase in BRCA1 mRNA levels in IM-9, there was no change in protein expression. These findings may point to a constitutive activation of the DNA damage pathway because of a persistent DNA damage in myeloma cell lines most likely based on defective DNA repair and apoptotic responses.

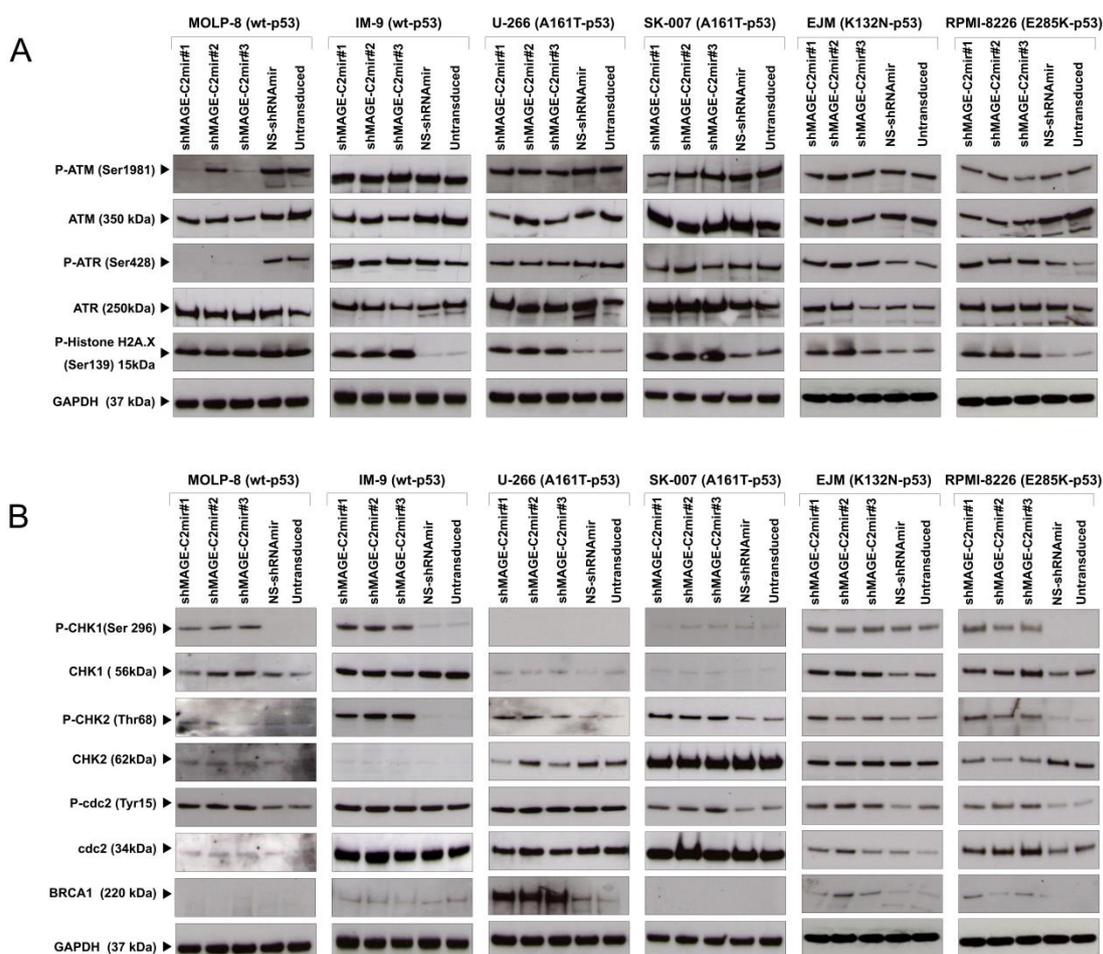


Figure 45. MAGE-C2/CT10 silencing enhances the activation of ATM/ATR-CHK1/CHK2 DNA damage pathway. (A) Western blot analysis of the phosphorylation status of major regulators of the DNA damage response, phospho-ATM (Ser1981), phospho-ATR (Ser428) and phospho-Histone H2A.X (Ser139) using the DNA Damage Antibody Sampler Kit (#9947, Cell Signaling) and the endogenous level of ATM ((D2E2), Rabbit mAb#2873, Cell Signaling) and ATR (#2790, Cell Signaling). (B) Western blot analysis of the phosphorylation status of proteins involved in the control of cell cycle and checkpoint in response to DNA damage response: phospho-Chk1 (Ser296), phospho-Chk2 (Thr68), phospho-cdc2 using the DNA Damage Antibody Sampler and Cell cycle/checkpoint Antibody Sampler Kit and their endogenous level: cdc2 (#9112, Cell Signaling), Chk1 (261D5, Mouse mAb#2360, Cell Signaling), Chk2 (D9C6, XP®, Rabbit mAb#6334, Cell Signaling)

CHAPTER 5: DISCUSSION

Despite significant advances in anti-myeloma therapies, MM remains an incurable disease because of the inevitable occurrence of resistance to treatments and disease progression. The development of immunotherapeutic strategies, to induce or to optimize an immune response capable of targeting and specifically eliminating tumor cells represents an alternative for the treatment of MM patients in relapse. Yet, it is widely believed, that successful cancer vaccines should preferentially target antigens possessing a vital role in tumor development and/or progression.

MAGE-C2, a member of the MAGE class I family of genes, is thought to be a good candidate for cancer immunotherapy given its very frequent expression in primary myeloma and immunogenicity. A number of studies support the premise that MAGE-C2 may interfere with the function of the tumor suppressor p53, which plays a pivotal role in activating the programmed cell death in response to oncogenic stimulation and DNA damaging agents. However, in spite of its frequent expression in MM, the biological role of MAGE-C2 in malignant plasma cells has never been elucidated and remains unclear.

This thesis focused on characterizing the functional role of MAGE-C2 in MM cell survival.

Using an RNA interference strategy, we investigated for the first time, *in vitro*, the effects of MAGE-C2 silencing on the viability of tumor cells derived from MM patients expressing functional and non-functional p53 in attempt to demonstrate whether the presumed anti-apoptotic effect of MAGE-C2 is strictly p53-dependent or not. Subsequently, the molecular basis of the presumed anti-proliferative and/or pro-apoptotic effects of MAGE-C2 in myeloma cells bearing functional and non-functional p53 were determined by a pathway-focused gene and protein expression analysis of key molecules involved in the positive and negative regulation of cell cycle progression, DNA damage, repair and apoptosis.

I. MAGE-C2/CT10 promotes proliferation and resistance to apoptosis in Multiple Myeloma

In this study, we have demonstrated for the first time that MAGE-C2 silencing impairs the proliferation and the viability of myeloma cells. The anti-proliferative effect of MAGE-C2 silencing was due to an inhibition of cell cycle progression at G0/G1 and/or G2/M, a decrease in the percentage of cells replicating DNA and an activation of apoptotic cell death. Anti-proliferative and/or anti-apoptotic effects of MAGE family members have been demonstrated in other tumors and have been mainly linked to the inhibition of the tumor-suppressing

function of p53. In human thyroid carcinoma, for instance, the enforced expression of MAGE-A3 resulted in an accelerated cell cycle progression with a decrease of cells in G0/G1 and an enhanced S phase entry. On the molecular level, these changes were due to a reduced level of p53-inducible cyclin-dependent kinase, p21^{WAF1/CIP1}, which is a key regulator of DNA damage-induced cell cycle arrest and inducer of the G1 arrest. Nevertheless, these effects were observed in cell lines bearing wild-type or mutant p53 suggesting a role of MAGE-A in modulating cell cycle arrest via p53-dependent and/or independent mechanisms [340]. On the other hand, while transient siRNA suppression of MAGE-A, -B and -C genes in mast cells had only modest effects on cell cycle progression and induced apoptotic cells death [273], stable MAGE-A knockdown in myeloma cell lines induced p53-dependent and independent apoptotic cell death but did not impair cell cycle progression [252]. These findings along with our results support a diverse role of MAGE class I proteins in the control of tumorigenesis by facilitating cell cycle progression and/or suppressing apoptosis and suggest that the biological effects of MAGE-C2 in comparison with other MAGE family members are not random but might be dictated by the cellular context.

We observed that the application of exogenous MAGE-C2 partially rescued the anti-proliferative effect of MAGE-C2 silencing and protected cells from apoptotic cell death; however, we still found a delayed progression through the G2/M. This finding suggests that MAGE-C2 may facilitate the entry into mitosis by suppressing apoptosis. Recently, another pro-survival mechanism for MAGE-C2 has been evidenced in the context of Melanoma demonstrating that MAGE-C2 may promote the growth and survival of malignant cells by inducing ATM kinase-dependent phosphorylation of KAP1-Ser824, which is a critical step in the cellular response to DNA damage to allow DNA relaxation and DNA repair. Inducible MAGE-C2 silencing in melanoma cells was associated with increased phosphorylation of histone H2A.X at ser139, which is a hallmark of DNA double strand breaks (DSB) and the ectopic expression of MAGE-C2 increased to 89% the repair of DSB by homologous recombination. Bathias' findings demonstrated for the first time the involvement of MAGE class I member in the DNA damage repair response by promoting the homologous recombination [276]. DSBs lesions are, in general, predominantly repaired in G1-phase by error prone non-homologous end-joining (NHJE), whilst in S-G2 phases the accurate homologous recombination (HR) seems to be the predominant DSB repair mechanism [341]. The involvement of MAGE-C2 in the HR DNA repair mechanism may provide an explanation for the pronounced delay in progressing across the S and G2/M phases following

MAGE-C2 silencing and even during the rescue assay. Besides the involvement of MAGE-C2 in DNA repair, few studies have attributed an anti-apoptotic role for MAGE-C2 by enhancing the E3 ubiquitin ligase activity of KAP1, which target p53 for proteasome degradation, suppressing thus p53-dependent apoptosis [216]. Accordingly, the silencing of MAGE-C2 is expected to lead to the accumulation and the stabilization of p53 protein, which is a central downstream checkpoint signalling protein in the DNA damage pathway responsible for cell cycle arrest and apoptotic response. These observations highlight the pleiotropic effect of MAGE-C2 in promoting cell cycle progression likely by enhancing DSB DNA repair at the S/G2/M phases and by suppressing p53-dependent and/ or independent apoptosis, which offers cancer cells a survival advantage in the presence of deleterious DNA damage such as DSB. DSB lesions can be introduced either endogenously by replication and oxidative stress or exogenously by DNA damage alkylating agents and when not repaired, DSB can induce an apoptotic signal that often fails to kill all damaged cancer cells because of apoptosis resistance [342].

II. MAGE-C2/CT10 promotes the clonogenic growth of Myeloma cells

The resistance to DNA damage-mediated apoptosis is a feature of cancer stem cells, which are often therapy-resistant and may cause clinical relapse [343]. In MM the existence of cancer stem cells has been demonstrated based on their unlimited self-renewal and clonogenic potential [132]. The drug resistance of myeloma stem cells has been attributed to a quiescent state which protects the tumor cells from DNA damage [132], an increased drug metabolism and efflux capability [132, 344] and, most importantly, the inactivation of apoptosis [345]. Accordingly, targeting of intracellular mechanisms that promote the survival of myeloma stem cells may be required to achieve prolonged remissions or even cures. Myeloma stem cells are present at very low levels in the bone marrow niche, the peripheral blood of MM patients [346], and in myeloma cell lines [132]. In our study, the colony formation assay showed that the transient and stable depletion of MAGE-C2 expression suppressed significantly the growth of clonogenic myeloma precursors in an anchorage-independent fashion, which is consistent with our previous findings showing that silencing of the closely related genes MAGE-C1/CT7 and MAGE-A3 impaired the clonogenic growth of myeloma precursors. These findings suggest that MAGE-C2 may contribute to the clonogenic potential of myeloma stem cells.

In tumorigenesis, the acquisition of the anchorage-independent growth is an important property of metastatic tumor cells as it allows them to escape cell-detachment induced apoptosis, migrate, and invade distant organs [347]. A number of studies have shown that the expression of MAGE proteins and other CTA is the highest in tumors with higher grade and metastatic potential suggesting that MAGE proteins may contribute to the aggressiveness of tumors by promoting their migratory and invasive potential [187, 188, 348]. Liu and colleagues provided plausible evidences that MAGE-A3 can modulate cancer progression by accelerating cell cycle progression, increasing cell migration rate, and promoting the development of lung metastasis in an orthopic model of thyroid cancer [340]. Furthermore, the expression of CT45, another CTA, was associated with disease progression and poorer outcome in Hodgkin's lymphoma and multiple myeloma [349, 350]. Koop and colleagues demonstrated that CT45 induced significant changes in the intra-cellular distribution of cytoskeleton-associated proteins thereby modulating cell adherence, morphology and migration, a phenomenon which may eventually contribute to disease progression [351]. It is well known that the alteration of the cytoskeleton modifies the baso-apical polarity as well as the shape of cells, which enables cells to migrate. The acquisition of migratory and invasive properties is associated with the activation of a transcriptional program conferring mesenchymal cell phenotype on tumor cells [352]. Epithelial Mesenchymal Transition (EMT) is an important biological event during embryogenesis which has also been linked to metastatic progression and drug resistance [353, 354]. A role for CTA in the EMT-related oncogenic behavior has been proposed because of their frequent expression in undifferentiated mesenchymal stem cells but not in their differentiated counterparts [206]. Furthermore, functional studies have shown that some CTA may promote cancer cell migration and chemoresistance by up-regulating key regulators of EMT, such as MMP2 and SNAIL [355], which represses a number of genes involved in p53-dependent apoptosis [356]. Interestingly, it has been shown that myeloma stem cells in general can be shed into the blood stream but only those cells that do not require anchorage-dependent growth can metastasize [357]. These combined observations and our findings that MAGE-C2 promotes the anchorage-independent growth of myeloma precursors, support the idea that MAGE family members and other CTA may sustain the metastatic behavior of cancer stem cells.

III. The molecular basis of the anti-proliferative and pro-apoptotic effects of MAGE-C2/CT10 silencing in MM

III.1 MAGE-C2/CT10 silencing induces p53-dependent cell cycle arrest and intrinsic apoptotic response

We have demonstrated herein that MAGE-C2 silencing in myeloma cell lines that express p53 with at least partial transcriptional function, induced an accumulation of p53 endogenous level. The accumulation of p53 protein was associated with an up-regulation of the negative regulators of G1/S and G2/M transitions namely CDKN1, GADD45A and CCNG1 and the down-regulation of key regulators of the mitotic spindle checkpoint, MAD2L1 and BIRC5, which may impair cell cycle progression and delay mitotic division. Knowing that p53 regulates positively CDKN1, GADD45A and CCNG1 expression and represses MAD2L1 and BIRC5, our findings suggest that MAGE-C2 mediated impairment of cell cycle progression was p53 dependent. Additionally, MAGE-C2 silencing induced the expression of the main down-stream mediators of p53-dependent intrinsic apoptotic pathway, the pro-apoptotic Bcl-2 genes BAX and BAK1 and the inhibition of the anti-apoptotic proteins Bcl-2 and MCL1 known to be negatively regulated by p53. All these findings demonstrate that MAGE-C2 may facilitate cells cycle progression and prevent apoptosis of myeloma cells by suppressing p53 transcriptional activity. The involvement of MAGE-C2 and other MAGE class I family members in the regulation of p53 anti-tumor function has been addressed in a number of studies and two mechanisms have been proposed based on the interaction of MAGE class I family members with KAP1 (TRIM28), that is a RING domain protein and a co-repressor of p53. In the first mechanism, MAGE expression enhances the formation of KAP1/p53 complex which promotes through the recruitment of histone deacetylase HDAC1, p53 deacetylation suppressing thus p53 transcriptional activity and conferring resistance to p53-dependent apoptosis in response to chemotherapeutic agents [217, 218]. In the second mechanism proposed recently by Doyle and colleagues, MAGE-C2 and other members of MAGE family were described to bind to KAP1 via the MAGE homology domain (MHD) which enhances the E3 ubiquitin activity of KAP1 and targets p53 for proteasome degradation. In line with these reports, our results did not show any increase of p53 mRNA level following MAGE-C2 silencing, suggesting that the accumulation of p53 endogenous level was regulated at the protein level likely by the inhibition of p53 degradation.

III.2 MAGE-C2/CT10 silencing activates E2F1-p73 pathway in the absence of functional p53

Despite the absence of a functional p53, MAGE-C2 silencing in EJM myeloma cell line induced cell cycle arrest and apoptosis in p53 similar manner likely through the activation of E2F1-TP73 apoptotic pathway. The hypothesis that MAGE-C2 may act as a regulator of E2F1 transcriptional activity is in line with previous observations showing that members of MAGE class II, NECDIN and MAGE-G1 interact with E2F1 transactivation domain which repressed E2F1-dependent transcription and antagonized E2F1-induced apoptosis of neuroblastoma cells [358, 359]. MAGE class II proteins /E2F1 transactivation domain interaction involves an amino acid stretch within the conserved MHD shared between all MAGE family members which may point to common regulatory mechanisms for instance the well documented suppression mechanism of p53-mediated apoptosis by MAGE I and II family members. Indeed, NECDIN and MAGE-A2 interact also with p53 transactivation domain via the MHD domain, thereby suppressing p21^{WAF1/CIP1} transcription and blocking p53-dependent apoptosis. In addition to the repression of p53 transactivation, MAGE-A2 may suppress p73 induced p21^{WAF1/CIP1} expression [217, 360, 361]. Given the high level of sequence homology among MAGE family members which could lead to functionally redundant proteins, we believe that MAGE-C2 may confer resistance to apoptosis in cancer cells, lacking functional p53, by suppressing the transcriptional activity of E2F1 and/ or p73 through protein/protein interaction involving the MHD.

Furthermore, it was previously shown that KAP1 represses E2F1 transcriptional and apoptotic activities by the recruitment of HDAC. Similarly to KAP1/p53 interaction, KAP1 binds to E2F1 via the coiled-coil region and in p53 null cells, silencing of KAP1 expression increases E2F1-mediated apoptosis in response DNA damage [362]. SIRT1 is a histone deacetylase that represses E2F1 and p53-dependent apoptosis in response to DNA damage and promotes cell survival. SIRT1 is recruited by NECDIN to suppress p53-dependent apoptosis in response to DNA damage [362-365]. On the basis of these evidences, we believe that, in addition to p53, E2F1 might be also a second target for MAGE-C2/KAP1 to suppress DNA damage mediated apoptosis in cancer cells lacking functional p53. Nonetheless, a biochemical analysis is required to confirm our observations and demonstrate the mechanism of E2F1 suppression by MAGE-C2.

III.3 MAGE-C2/CT10 silencing enhances the ATM/ATR-CHK1/CHK2 DNA damage response

Our analysis for the activation status of a number of proteins involved in cell cycle and checkpoint control revealed that the loss of MAGE-C2 expression was associated with an increased phosphorylation of the histone variant H2A.X at Ser139 which is a sensitive indicator of DSB. We also showed a constitutive phosphorylation at Ser1981 and Ser428, respectively, of the primary sensors of DNA damage ATM and ATR, independently of MAGE-C2 silencing, suggesting the presence of an ongoing DNA damage in myeloma cell lines. Downstream of ATM and ATR, MAGE-C2 silencing enhanced the activation of CHK2 and/or CHK1 through an increased phosphorylation at Ser296 and Thr68, respectively, as well as the induction of p53 phosphorylation at Ser20 residue resulting into the transcriptional activation of p53. Altogether, these observations demonstrate a constitutive activation of the ATM/ATR-CHK1/CHK2 DNA damage pathway. MAGE-C2 silencing seems to enhance the activation and the transduction signal of the DNA damage response by the stabilization of p53, which plays a central role in connecting the DNA damage pathway to the apoptotic machinery in the presence of irreversibly damaged DNA. In the absence of functional p53, the DNA damage pathway may also induce an apoptotic signal by activating the E2F1, a common substrate to CHK1 and CHK2, which induces E2F1 stabilization, transactivation, leads to the up-regulation of the pro-apoptotic protein p73 and consequently to the activation of apoptosis in p53 similar manner. The CHK1/CHK2-E2F1-p73 pathway may constitute an alternative apoptotic pathway when p53 function is altered [366] that can be suppressed by MAGE-C2 to promote cell survival in the presence of DNA damage such as DSB.

In tumor cells, DSB can arise either from exogenous insults such as ionizing radiations and alkalyting agents or from endogenous insults such as oncogene-mediated replication stress. Activated oncogenes such as RAS may contribute to the formation of DSB by inducing hyperproliferative oncogenic stimuli leading to unscheduled S phase entry and to a massive interference between replication and transcription which results into the collapse of the replication fork, the accumulation of DSB, chromosomal instabilities and the induction of constitutive activation of the DNA damage response [367-369]. Evidences of ongoing DNA damage in malignant plasma cells from MM patients and in human myeloma cell lines were previously demonstrated by the presence of a constitutive phosphorylation of the histone protein H2AX, ATR, CHK1 and CHK2 in the absence of any DNA damaging treatments which might be indicative of oncogene mediated replication stress [370]. In multiple myeloma,

the acquisition of activating mutations in the oncogenes K-Ras, N-Ras is an important transforming event in the evolution of MGUS to MM (20-35%) and occurs in the majority of Plasma cell leukaemia patients (63-70%) which results in growth factor independence and suppression of apoptosis. However, the persistent mitogenic stimulation of Ras oncogenes could promote replication stress and constitutive activation of DNA damage response [371-373]. It is believed that continuous activation of DNA damage pathway in malignant cells may select for those cells containing p53 mutations or deletions, to allow cells bypass the cell cycle checkpoints and continue cycling even in the presence of DNA damage. Although, mutated p53 confers survival advantage, some tumors such as bone, testis and MM do not frequently mutate p53 but express proteins that target wt-p53 protein function by diverse mechanisms [260, 261]. In MM, although transforming events lead to the proliferation and survival of terminally differentiated plasma cells, programs that control plasma cell differentiation and B-cell gene inhibition appear to be largely intact like p53 transactivation which is required for cell cycle exit during B cell differentiation [374]. It might be that malignant plasma cell express proteins such as MAGE-C2 that do not suppress tightly the function of p53 leaving a residual activity that maintains the differentiation state as evidenced by the basal level of p53 endogenous protein level itself and its transcriptional target p21^(WAF/CIP1) that halts or slows cell cycle progression. This loose inhibitory activity might be compensated by a second mechanism up-stream the DNA damage-p53 pathway. Bathia and colleagues have shown that MAGE-C2 interacts with KAP1 to enhance p53 degradation and to increase ATM-dependent phosphorylation of KAP1 at Serine 824 in response to DNA damage, which leads to DNA decondensation at DSB sites within transcriptionally silent chromatin, allows DNA repair by homologous recombination and rescues cell from cell death [276, 375]. Furthermore, the silencing of MAGE-C2 in A375 melanoma cells induced the phosphorylation of H2A.X, which is in agreement with our findings and can be indicative of DSB occurrence following MAGE-C2 silencing. These functional evidences demonstrate that, besides its anti-apoptotic activity, MAGE-C2 may promote the growth of transformed cells by enhancing DNA damage repair in heterochromatin.

Defect in the DNA repair machinery is a feature of cancer cells that gives rise to mutations and somatic rearrangements, promotes abnormal proliferation and drives the neoplastic transformation [376]. Nonetheless, to replicate their DNA, cancer cells have to fix damaged DNA by inaccurate alternate DNA repair pathways such as the error prone alternate non-homologous end-joining (NHEJ) regulated by PARP-1 whose the inhibition (PARP inhibitor)

constitutes a highly effective therapy for cancer cells [377, 378]. Homologous recombination also includes an inaccurate alternate pathway that competes with NHEJ for DSB repair at S-G2/M phases [379] and whose the efficiency might be enhanced by proteins exclusively expressed by cancer cells, such as MAGE-C2.

IV. CTA, the DNA damage and apoptotic responses in spermatogenesis

Although the occurrence of DSB breaks can destroy the genome, it constitutes a natural event required for a number of physiological processes namely V(D)J and IgH class switch recombination in lymphoid cells, chromatin remodelling and changes in gene expression involved in information processing, learning and memory in post-mitotic neurons, and most importantly meiotic recombination in germ cells [380-383]. Interestingly, in male meiotic recombination, the DSB are induced by the Non-X CTA SPO11 and SCP1 forming the synaptonemal complex that mediates chromosome pairing, synapsis and recombination [196, 197]. Several other Non-X CTA have been described to be involved in the regulation of meiosis in testis and their aberrant expression in cancer is thought to generate aneuploidy, a feature shared between cancer and germ cells [172]. In contrast, the majority of X-CTA such as MAGE family members are predominantly expressed in pre-meiotic germ cells. Because male mice expressing mutant Kit tyrosine kinase (c-kit), known to regulate MAGE gene expression, are infertile due to increased germ cell apoptosis, MAGE proteins are likely to promote the survival of pre-meiotic germ cells. However, different expression pattern of X-CTA have been observed for MAGE-C2 [172, 266]. While MAGE-C2 is weakly expressed in spermatogonia, primary spermatocytes show a strong MAGE-C2 expression. At later stages of maturation, MAGE-C2 expression gradually decreases and becomes undetectable in mature spermatids [265, 266]. Like in advanced grade tumor cells, MAGE-C2 shows a nuclear sub-cellular localization in spermatogonia and spermatocytes. The nuclear expression of MAGE-C2 in primary spermatocytes that coincides with the first meiotic division may imply a role in the regulation of the meiotic events by a mechanism shared in common between primary spermatocytes and advanced grade tumor cells [265, 268, 269]. One likely mechanism is the regulation of DSB repair and p53-dependent apoptosis which are two important pathways in cancer and primary spermatocytes.

Although the induction of DSB during the first meiotic division contributes to the maintenance of genomic integrity, it may make primary spermatocytes vulnerable to DNA

damage, which highlights the importance of the activation of the DNA damage response during spermatogenesis. In non-irradiated adult testis the marker of DSB, γ H2A.X is detected, during the first meiotic prophase, in leptotene and early zygotene spermatocytes, which is equivalent to the S-G2 transition in mitosis [384]. During the pachytene stage, which is equivalent to the G2/M mitotic checkpoint, these DSB are repaired [385]. However, in case of incomplete resolved DSB, meiotic division may fail and damaged cell may die. Nonetheless, despite the presence of DSB, which may in other cells trigger DNA damage mediated apoptosis, if not instantly repaired; the DNA replication of spermatocytes in the pre-meiotic S phase is not interrupted. These observations suggest a tight and coordinated regulation of the DNA damage, DNA repair, cell cycle progression and apoptosis. The DNA repair mechanism in pachytene spermatocytes is induced by activated ATM kinase, which targets many proteins (CHK1, CHK2) to coordinate cell cycle arrest to DNA damage repair via homologous recombination and to inhibit apoptosis [386-389]. Indeed, men with ataxia telangiectasia and mice lacking ATM gene, are infertile because of extensive apoptosis of damaged spermatocytes following a failed meiosis [390]. Besides the DNA damage response, the apoptosis is also essential in spermatogenesis for the regulation of germ cell density and for the selective removal of damaged germ cells. The tumor suppressor gene, p53 has been proven to play a central role in DNA damage induced spermatogonial apoptosis [391, 392]. In primary spermatocytes, p53 is expressed in the absence of irradiation, which suggests a role for p53 in the regulation of the first meiotic prophase. However, unlike spermatogonial cells that undergo spontaneously apoptosis, spermatocytes undergoing meiotic division are much more resistant to apoptosis, because p53 appears to be much more involved in the DNA repair during homologous recombination than in apoptosis [393]. It is likely that p53 and ATM are activated at low level or for a short time to deal with any DNA damage and avoid thus any massive activation of the DNA damage response that may lead to male germ cell death. Moreover, spermatocytes that accumulated DNA damage due to abnormal chromosomal synapsis may undergo p53-independent apoptosis mediated by E2F1 [394] which appears to play a role in the control of germ cell fate. Both E2F1 overexpression and depletion in mice testis cause an excessive germ cell apoptosis [395], [396]. Like p53, E2F1 is also a target for activated ATM Kinase during the DNA repair mechanism operated during the pachytene stage [397]. Nonetheless, in normal pachytene spermatocytes, E2F1 translation seems to be inhibited to prevent E2F1 mediated apoptosis during meiotic recombination [398]. Although less efficient than p53 route, the pro-apoptotic p73 is expressed in spermatocytes at the nucleus which suggests a role in controlling genome integrity during meiosis [399]. All these

mechanisms for apoptosis inhibition whether p53-dependent or independent that accompanied meiotic recombination can be regulated by sex specific factors such as CTA to rescue cells from the deleterious effects of DSB and can be hijacked by cancer cells to facilitate cell cycle progression and escape apoptosis despite aneuploidy.

V. A potential role for MAGE-C2/CT10 in the DNA damage and apoptotic responses

In the present study, we have shown that irrespective of p53 status, MAGE-C2 silencing in malignant plasma cells delayed cell cycle progression through S-G2/M phases mainly because of an increased expression of p21^{WAF1/CIP1} and GADD45 α , which are two major inhibitors of cell cycle progression in response to DNA damage. Furthermore, we found a constitutive activation of the DNA damage response in myeloma cells which is indicative of a pre-existing DNA damage. MAGE-C2 silencing enhanced further the activation of the DNA damage response as evidenced by an increased phosphorylation of γ H2AX, CHK1 and/or CHK2, cdc2, an up-regulation of many genes involved in the DNA damage and repair responses as well as an activation of p53-dependent and independent intrinsic apoptotic pathway. These observations may support a role of MAGE-C2 in the regulation of DNA damage, repair and apoptotic responses, which may facilitate cell cycle progression and mitotic division of cancer cells. This putative role of MAGE-C2 may be operated in healthy cells to facilitate meiotic progression of male germ cells despite aneuploidy. The same mechanism could be engaged by cancer cells to escape cell death and enforce mitotic progression despite DNA repair defect and genetic instability. Additionally, finding that MAGE-C2 depletion suppressed the expression of a number of genes regulating the mitotic checkpoint to prevent aneuploidy, points to a role of MAGE-C2 in the regulation of chromosomal segregation. Despite the lack of functional evidences, an involvement of Cancer-testis proteins in the regulation of the SAC is likely highlighted particularly because of the frequent expression of these proteins in solid tumors and in MM, among haematological malignancies [400] which are characterized by a highly instable genome not only limited to chromosome translocations but extend to chromosomal loss and gain. In fact, it is this complex chromosomal instability that distinguishes Multiple Myeloma from the other hematological malignancies which is likely attributed to mitotic spindle checkpoint dysfunction and DNA repair defect [401, 402].

**CHAPTER 6: SUMMARY,
CONCLUSIONS AND PERSPECTIVES**

Summary, conclusions and perspectives

Despite significant improvements in the treatment of MM, the median survival has not improved beyond 4-5 years and relapse will eventually occur in over 90% of patients initially responding to treatments. There is ample evidence that the drug resistance is due to the persistence of a small population of malignant plasma cells displaying stem cell properties including chemoresistance and clonogenic growth. Therefore, the selective targeting of intracellular mechanisms crucially involved in promoting the survival of these resilient cells may lead to the eradication of both myeloma precursors as well as their more differentiated progenitors.

Cancer-testis antigens belonging to MAGE class I family of genes are commonly expressed in MM. Expression of MAGE class I genes is associated with an aggressive clinical course of MM and resistance to chemotherapy, suggesting that MAGE genes may confer a survival advantage on myeloma cells. MAGE-C2 is a member of the MAGE class I family of genes thought to be a good candidate for cancer immunotherapy given its very frequent expression in primary myeloma. A number of studies support the premise that MAGE-C2 may interfere with the function of the tumor suppressor p53, which plays a pivotal role in activating the programmed cell death in response to oncogenic stimulation and DNA damaging agents. However, in spite of its frequent expression in MM, the biological role of MAGE-C2 in malignant plasma cells has never been elucidated.

Therefore, by using the RNA interference strategy, we investigated for the first time, *in vitro*, the effects of MAGE-C2 silencing on the viability of tumor cells derived from MM patients expressing functional and non-functional p53, in attempt to demonstrate whether the presumed anti-apoptotic effect of MAGE-C2 is strictly p53-dependent. Subsequently, the molecular basis of the presumed anti-proliferative and/or pro-apoptotic effect of MAGE-C2 in myeloma cells bearing functional and non-functional p53 was determined by pathway-focused gene and protein expression analyses of key molecules involved in the positive and negative regulation of cell cycle progression, DNA damage, repair and apoptosis.

We found that loss of MAGE-C2 expression resulted in reduced proliferation, viability, and anchorage-independent growth of myeloma cell lines, irrespective of the functional status of

p53. The anti-proliferative effect of MAGE-C2 silencing was due to a decrease of cells in the S phase, a cell cycle delay at both G0/G1 and/or G2/M transitions, and an increase in the subG0/G1 diploid population based on an initiation of apoptotic cell death. Importantly, MAGE-C2 overexpression was able to rescue the anti-proliferative effect of MAGE-C2 depletion and protected cells from apoptotic cell death.

At the molecular level, the loss of MAGE-C2 expression was associated with an increase in the phosphorylation of the histone variant H2A.X on Ser139 which is a sensitive indicator of DNA damage. A constitutive phosphorylation on Ser1981 and on Ser428, respectively, of the primary sensors of DNA damage ATM and ATR were detected independently of MAGE-C2 silencing suggesting the presence of an ongoing DNA damage in MM cell lines. Downstream of ATM and ATR, MAGE-C2 silencing was associated to the activation of CHK2 and/or CHK1 through phosphorylation at Ser296 and Thr68, respectively, and an increase in the endogenous level of p53 protein. The stabilization and activation of p53, through phosphorylation at Ser20 by CHK1 and CHK2, correlated with an up-regulation of two transcriptional target genes, p21^(WAF1/CIP1) and GADD45A, which are the main mediator of p53-dependent and independent G1/S and G2/M cycle arrest, respectively, and two-fold increase in the expression of the pro-apoptotic Bcl-2 genes BAX and BAK1, being the effectors of p53-dependent and independent intrinsic apoptotic pathway in response to DNA damage.

Taken collectively, we demonstrated in this thesis, that MAGE-C2 promotes the survival of myeloma cells by facilitating cell cycle progression, suppressing apoptosis and enhancing the anchorage-independent growth of myeloma precursors. The analysis of the molecular mechanism underlying MAGE-C2 silencing mediated effects, strongly, supports an anti-apoptotic function of MAGE-C2 in MM through the regulation of key molecules involved in the DNA damage repair pathway, p53-dependent and independent apoptosis. Accordingly, the aberrant expression of MAGE-C2 expression in MM cells may contribute to one of the molecular aspects that drive the complex behaviour of malignant cells namely the DNA repair and resistance to apoptosis in response to DNA damage, regardless of p53 functional status. These findings are in agreement with prior observations reporting that MAGE class I expression in MM and other tumors correlates with resistance to chemotherapeutic agents, *in vitro* and *in vivo*. The potential role of MAGE-C2 in the DNA repair and apoptotic responses may constitute a new therapeutic route that can be targeted in order to potentiate the cytotoxicity of chemotherapeutic agents to which most MM patients acquire resistance.

Yet, further functional analysis in both carcinogenesis and spermatogenesis are needed for the design of efficient therapeutic tools that target DNA repair mechanisms sustaining cancer cell proliferation and resistance to chemotherapeutic agents

Knowing that HMCL are not cultured in contact with BMSC and still, unlike, primary myeloma cells, they display higher proliferative rate, we acknowledge that *in vitro* models cannot recapitulate the complex behaviour of MM cells, intimately dependent on the bone marrow microenvironment niche that confers survival signals and resistance to drugs. It is, therefore, important to conduct future studies on primary myeloma cells derived from patients and *in vivo* to gain much more insights into the impact of the depletion of MAGE-C2 or even collectively with other demonstrated anti-apoptotic MAGE class I proteins on the behaviour of malignant plasma cells. Unlike other hematological malignancies, MM is a heterogeneous disease because of lack of any fundamental driving molecular aberration, therefore the design of combinatory treatment strategies targeting several MAGE proteins in combination with chemotherapy might be effective in circumventing or reverting drug resistance.

To our knowledge, this study demonstrates, for the first time, a new anti-apoptotic mechanism promoting the survival of myeloma cells and perhaps conferring drug resistance which remains to date the most challenging aspect in the treatment of MM patients. The central role of MAGE-C2 in the biology of myeloma suggests that this CTA represents a promising target for myeloma-specific immunotherapies or other targeted modes of therapy for MM.

CHAPTER 7: REFERENCES

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