Regulation of Tumorigenic Spliced Isoforms in Cancer

THESIS

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By

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Abstract

MDM2 negatively regulates the tumor suppressor protein p53 by blocking its transcriptional activation domain and targeting p53 for proteosomal degradation. Upon induction of specific DNA damage and in cancer the MDM2 transcript undergoes drastic exclusion of its internal exons through the process of alternative splicing. This damage induced alternative splicing results in the generation of the most common alternative spliced form identified in human cancers, MDM2-ALT1. As opposed to full length MDM2, MDM2-ALT1 lacks the p53 binding domain which renders this protein isoform incapable of an interaction with the tumor suppressor. However, our lab and others have shown that MDM2-ALT1 is capable of binding full length MDM2 and to sequester it to the cytoplasm. This MDM2-MDM2-ALT1 interaction inhibits the negative regulation exerted by MDM2 over p53. Therefore, MDM2-ALT1 through its interaction with MDM2, mediates a protective program in which p53 is stabilized and cell cycle arrest, DNA repair and/or apoptosis is induced as a consequence. Because the observed role of MDM2-ALT1 in promoting cell proliferation inhibition is inconsistent to its extensive detection in human cancers, we decided to
investigate the regulation behind the alternative splicing of MDM2 and also to determine its role in tumorigenesis. We have identified positive and negative exonic splicing regulators of MDM2 using a system of MDM2 minigenes that upon induction of DNA damage parallel the alternative splicing events that take place in a cancer cellular context in which the generation of MDM2-ALT1 is characteristic. To identify the role of MDM2-ALT1 in tumorigenesis we have generated an in vivo mouse model in which the expression of MDM2-ALT1 is restricted to B cells. We report that mice expressing the MDM2-ALT1 transgene show a significant prevalence in the emergence of degenerative joint disease, an inflammatory disorder associated to the clonal expansion of B cells. Further characterization of the expression of MDM2-ALT1 in B cells using our mouse model is still to prove its usefulness in elucidating the role of MDM2-ALT1 in tumorigenesis.
Dedicated to my son Gerardo Alejandro and my family for their loving support

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Chapter 1: Introduction

1.1 Constitutive and Alternative pre-mRNA Splicing

Gene expression begins with the transcription of a gene from DNA to pre-
mRNA. In eukaryotes, almost all protein coding genes will generate a pre-mRNA
in which the coding sequences called exons are separated by non-coding
sequences called introns. Pre-mRNA splicing takes place co-transcriptionally to
yield an mRNA, in which the introns are precisely excised and the exons are
accurately joined. Altogether with 5’ capping and 3’ polyadenylation, pre-mRNA
splicing is a crucial regulatory step in the generation of a mature mRNA molecule
that can be exported to the cytoplasm for translation into a protein (Fig. 1).

Most exons are constitutive; they are always spliced or included in the
final mRNA (BLACK 2003). However, in alternative splicing a regulated exon can
sometimes be included and sometimes excluded to produce a final transcript that
is different to the one that results from constitutive splicing (Fig. 1). Differential
inclusion or exclusion of coding sequences in the final mRNA through alternative
splicing results in the generation of protein isoforms with different biological
functions that differ in protein:protein interaction, subcellular localization or
catalytic ability (STAMM et al. 2005). Therefore, alternative splicing is a key regulator of gene expression that also contributes to proteome complexity.

1.2 The splicing reaction and the spliceosome

The splicing reaction involves a two step biochemical process in which single transesterification reactions occur through the recognition of conserved consensus sequences at the intron-exon junction, called splice sites. These consensus sequences are known as the core splicing signals and are the 5’ splice site, the 3’ splice site and the branch point sequence. The 5’ splice site refers to the exon-intron junction at the 5’end of the intron and is characterized by the nearly invariable dinucleotide GU (MOUNT 1982). The 3’ splice site is found at the 3’ end of the intron and it contains three conserved sequence elements, those are the branch point, the polypyrimidine tract and an AG dinucleotide (Fig. 2A). The first step is the cleavage of the 5’ splice site and lariat formation (KRAINER et al. 1984; RUSKIN et al. 1984). In this step the 2’- hydroxyl group of an adenosine nucleotide at the branch point attacks the phosphate at the 5’ splice site, resulting in the excision of the 5’ exon from the intron and in the ligation of the intron 5’ end to the branch point 2’ hydroxyl. Two intermediates are generated as the result of this reaction: a free 5’ exon and an intron-3’ exon fragment in a lariat configuration (BLACK 2003; BURGE et al. 1999). In the second step, 3’-hydroxyl group from the free exon attacks the phosphate at the 3’ splice site giving rise to the ligation of the two exons and the release of the intervening intron in an intact lariat RNA (BURGE et al. 1999; PADGETT et al. 1986) (Fig. 3).
The biochemical splicing reactions described above are driven and catalyzed by the spliceosome, a dynamic complex of small nuclear RNAs (snRNAs) and over 140 proteins that assembles in the pre-mRNA molecule by recognition and base pair interaction with the conserved consensus sequences embedded in the splice sites (Zhou et al. 2002). The spliceosome is composed by five small nuclear RNA protein complexes (snRNPs) in which the U1, U2, U3, U4, U5 and U6 snRNAs interact with an array of proteins that can be snRNP specific or common to each snRNP. These common proteins are called Sm proteins and they are key players in facilitating the kinetics of the snRNP assembly while providing thermodynamic stability to the complex (Raker et al. 1999). The assembly of the spliceosome takes place in a stepwise fashion and it is subject to different conformational changes to give rise to the specific complex that is required at a particular stage of the splicing reaction. Initial recognition of the intron 5’ and 3’ splice sites is driven by the E (early) or commitment complex. The E complex is formed through the ATP independent binding of the U1 snRNP to the 5’ splice site via base pairing interactions, altogether with binding of the SF1/BBP protein to the branch point, the U2 auxiliary factor 65 (U2AF65) to the polypyrimidine tract and U2AF35 to the 3’ splice site (Graveley et al. 2001). The binding of SF1 and U2AF proteins is important for the up-coming stable association of the U2 snRNP with the pre-mRNA in the formation of the A complex (Graveley et al. 2001; Query et al. 1997). In the A complex, the U2 snRNP binds to the pre-mRNA BPS through base pairing interactions that are stabilized by the proteins SF3a, SF3b and U2AF65 and that result in the
displacement of SF1/BBP from the BPS (Gozani et al. 1996; Query et al. 1997; Valcarcel et al. 1996). The assembly and the dissociation of the A complex is ATP dependent as is the subsequent formation of the B complex. The U4/U6 and U5 snRNPs are recruited to the pre-mRNA as a pre-assembled but inactive B complex (Konarska and Sharp 1987; Utans et al. 1992). Complex A associates with complex B through an interaction between the U2 snRNP and U5 snRNP that results in the positioning of the last one at the 3' splice site (Chiara et al. 1997; Konarska and Sharp 1987). Other major conformational and compositional arrangements take place and result in the destabilization and release of U1 and U4 snRNPs, positioning of U6 snRNP at the 5' splice site and catalytic activation of the complex (Konarska and Sharp 1987; Konarska and Sharp 1988; Lamond et al. 1988). With the release of U1 and U4 snRNP the now active C complex catalyzes the first of the splicing reaction. For the C complex to carry the second splicing step, repositioning of the intermediates generated in the first step at the catalytic center of the spliceosome needs to occur. Also, additional rearrangements of the snRNPs, like destabilization of the U6 snRNP interaction with the 5' splice site are required for the second step of splicing (Konarska et al. 2006). After completion of the second splicing step, the spliceosome dissociates, releases and makes U2, U5 and U6 available for subsequent rounds of splicing and a fully spliced mRNA gets generated (Fig. 4) (Licht et al. 2008). Consequently, spliceosome assembly is a highly regulated mechanism that is dependent on the chronological establishment of several spliceosomal complexes at specific stages during the splicing process with the final aim of
catalyzing the biochemical reactions that will generate a fully spliced and mature mRNA.

1.3 Regulation of splicing through cis-acting elements and trans-acting factors.

Given the biological relevance of splicing and alternative splicing in gene expression it is expected that these processes are under the control of highly regulated mechanisms that will minimize mistakes in the synthesis of proteins. The core splicing signals described before only provide an initial level of regulation by which the spliceosome machinery can be recruited to the pre-mRNA’s specific sites. At the same time, the unique conditions and requirements under which the spliceosome assembles to catalyze the splicing reactions also contribute to the regulation of the process. However, it is hard to imagine that the spliceosome machinery can easily distinguish the core splicing signals in the multitude of nucleotides (over 100kb) that makes up an intron solely by recognizing the nearly invariant dinucleotides at the splice site and the branch point sequence. Indeed, in order to distinguish an exon from a pseudoexon (an exon size sequence that contains apparent splicing signals) and avoid the synthesis of aberrant proteins that can cause disease, an additional layer of regulation is exerted by regulatory cis-acting elements in the pre-mRNA and the trans-acting factors that bind them. The interactions that arise between these regulatory elements allow for precise exon/intron definition in constitutive splicing
and moreover, they provide a mechanism by which the generation of different splicing isoforms is regulated by alternative splicing.

The regulatory *cis*-acting elements can be classified based on their location in the pre-mRNA and by their role into either promoting or inhibiting the splicing reaction at a given site. Exonic splicing enhancers (ESEs) and intronic splicing enhancers (ISEs) are purine-rich sequences that stimulate splicing and are accordingly located at the exons or intron. These enhancer sequences function by recruiting splicing factors that will in turn guide the spliceosomal components to the splice sites. Conversely, exonic splicing silencers (ESS) and intronic splicing silencers (ISSs) promote the binding of silencing factors that will block splice sites recognition, therefore repressing splicing (Fig. 3B).

Members of the SR protein family recognize the ESE and ISE regulatory sequences. SR proteins are essential splicing factors that have one or two N-terminal RNA recognition motifs (RRM) that allow for RNA interaction and provide substrate specificity followed by a serine/arginine rich domain at the C-terminal (RS domain) that promotes protein:protein interactions (BIRNEY et al. 1993; Fu 1995). The RS domain also plays a role in the RNA:protein interaction since serine phosphorylation can alter the RNA binding of SR proteins *in vitro*, as a mean to avoid non specific interactions with RNA (TACKÉ et al. 1997). The exon definition function of SR proteins is achieved by mechanisms that are not mutually exclusive and that are dependent or independent of their RS domain. First, in the RS independent mechanism, SR proteins regulate exon definition via their RRM which can provide a surface for protein:protein interaction that can
make up for those mediated by the RS domain. SR proteins can also promote exon definition under this mechanism by competing with silencing factors via an RNA:protein interaction with the pre-mRNA that antagonizes adjacent silencer elements (ZHú and Krainer 2000) (ZHú et al. 2001). Second, through an RS domain dependent mechanism, SR proteins establish the protein:protein interactions that recruit the splicing machinery to the splice sites. It has also been showed that the RS dependent mechanism of action of SR proteins is critical in promoting splicing when the splicing signals in the pre-mRNA are weak (Tacke et al. 1997; ZHú and Krainer 2000).

Several online prediction programs have been developed to assess the potential of a splicing factor for binding to a particular ESE sequence. One such program is ESE finder which provides a predicted score value for the binding of 5 individual SR proteins to the sequence of interest (Cartegni et al. 2003; Smith et al. 2006). These predictions are based on experimental data developed through a systematic evolution of ligands by exponential enrichment (SELEX) approach and they have proven to be an important tool in understanding how the exon definition mechanism is regulated (Cartegni et al. 2003; Smith et al. 2006).

The ESSs and ISSs regulatory sequences are recognized by heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNP’s have one or two RRM’s and a glycine rich domain that is involved in protein:protein interactions. The mechanisms by which ESS/ISS and hnRNPs negatively regulate splicing are not as characterized as ESE/ISE and SR proteins regulatory mechanisms. However, proposed mechanisms have emerged from the study of hnRNP A1 and
Polypyrimidine Tract Binding Protein (PTB), two important splicing repressors. It has been shown that hnRNP A1 can inhibit splicing through a high affinity binding interaction with a silencer sequence that in turn promotes cooperative binding and nucleation of hnRNP’ A1s in the pre-mRNA. This results in blockage of sequences adjacent to the silencer as well as adjacent enhancer sequences to which SR proteins would bind (ZHOU et al. 2001). In the other hand, PTB promotes splicing inhibition by antagonizing the formation of the spliceosomal E complex while competing for the binding site of U2AF at the polypyrimidine tract. Also, PTB can mediate splicing repression by “looping out” an exon that is flanked by silencer sequences to which PTB can bind (WAGNER and GARCIA-BLANCO 2001). Binding of PTB at these sequences makes the looped exon unreachable to the splicing machinery.

Overall, the relevance of both cis- acting regulatory sequences and trans-acting factors in the regulation of splicing is highlighted by compounding evidence that show that the alteration of enhancer and silencer sequences is associated with genetic disorders, altered regulation of cancer susceptibility genes and oncogenic signaling and by studies showing that the function and localization of some of these splicing factors can also be determining in the emergence of many diseases (COLAPIETRO et al. 2003; DAVID and MANLEY 2010; KALNINA et al. 2005; LIU et al. 2001; SHEPARD and HERTEL 2009; ZATKOVA et al. 2004)
1.4 Splicing and alternative splicing as a cause of disease.

It has recently been shown by an in-depth analysis of deep sequenced human DNA that 92-94% of the human genes undergo alternative splicing (WANG et al. 2008). This finding explains how approximately 100,000 different proteins could be synthesized through the transcription of the estimated 24,000 protein coding genes that constitute the human genome (MODREK and LEE 2002). Therefore, because the mechanism of alternative splicing is a major contributor to protein diversity, disruption if this process at any of the regulatory stages that govern it, can have serious implications in the emergence of disease by the expression of aberrant or misregulated proteins. In line with this, it has been estimated that 50-60% of the disease causing mutations affect or disrupt splicing (CARTEGNI et al. 2002; LÓPEZ-BIGAS et al. 2005; PAGENSTECHER et al. 2006). Moreover, from the mutations recorded in the Human Gene Mutation Database (HGMD) that are not embedded in splice sites, over 75% alter exonic sequences either by single-nucleotide substitutions, micro deletions and/or insertions (STENSON et al. 2003).

Disease-causing disruptions of constitutive and alternative splicing result from alterations of cis- acting elements and/or trans- acting factors. In the case of cis- acting elements these disruptions are caused by mutations in splice sites, silencers, enhancers and/or by insertions and deletions that can either generate or abrogate a binding site. In the other hand, mutations of trans- acting factors or changes in their concentration or localization can well promote changes in splicing patterns that lead to adverse consequences to human health. Indeed,
there is convincing evidence about how anomalies in these splicing regulatory elements and factors can lead to the emergence of disease. For example, from the 1388 mutations identified in the cystic fibrosis transmembrane conductance regulator gene (CFTR), 185 of them are splicing mutations that have been identified as 25 being exonic and the remaining being intronic (Novoyatleva et al. 2006). Complete skipping of CFTR exons 9 and 12 occurs due to several splice-site mutations and it results in the clinical onset of cystic fibrosis, where patients suffer chronic respiratory and digestive problems (Hull et al. 1994). In spinal muscular atrophy (SMA), a neurodegenerative disorder with progressive paralysis caused by the loss of alpha motor neurons in the spinal cord, the SMN1 gene that encodes the SMN protein that regulates snRNP assembly is lost. Even though humans carry an almost identical gene, SMN2, a silent C>T change at position 6 in exon 7 results in exclusion of this exon and in a truncated protein that cannot compensate for the lack of SMN protein that should have been generated by the SMN1 gene. The splicing factor PRPF31 is required for stable assembly of the U4/U6.U5 tri-snRNP and mutations in this gene have been identified in patients who suffer autosomal dominant retinitis pigmentosa, a progressive degeneration of the retina that can lead to blindness. It is now known that mutant PRPF31 protein causes apoptosis of retinal cells by inhibiting the splicing of intron 3 of the RHO transcript, which expresses a G-protein-coupled receptor activated by light that initiates the phototransduction cascade that converts light signals to electrophysiological signals in retinal neurons (Yuan et al. 2005). All of the above highlight the relevance of understanding splicing and
alternative splicing regulation in the context of individual genes as a way to identify disruptions that result in disease. Most importantly is the fact that a thorough understanding of these processes could lead to the development of novel therapeutic approaches that can target splicing as the main causative of human maladies.

1.5 The MDM2-p53 pathway

The murine double minute 2 gene (mdm2) was discovered as an amplified gene found on double minute chromosomes in a spontaneously transformed BALB/c mouse cell line identified as 3T3DM (CAHILLY-SNYDER et al. 1987). The amplification of this gene was then associated with transformation which comes as a result of Mdm2 binding interaction with the tumor suppressor protein p53 (FAKHARZADEH et al. 1991; MOMAND et al. 1992). Binding of Mdm2 to p53 inhibits the tumor suppressor trans activation domain and therefore the transcriptional activation of its downstream targets (MOMAND et al. 1992). Homologues of Mdm2 are present in other species and in humans the MDM2 gene has been mapped to the 12q13-14 chromosome (OLINER et al. 1992). The Mdm2-p53 interaction observed in mice turned out to be conserved in humans since in vitro binding experiments using recombinant human MDM2 resulted in binding to p53 and the expression of MDM2 in yeast inhibited human p53 ability to transactivate downstream gene targets. (OLINER et al. 1992; OLINER et al. 1993). MDM2’s role as a p53 regulator is not limited to its ability to block the tumor suppressor
transactivation domain, since it also targets p53 for proteasomal degradation by performing as an E3 ubiquitin ligase (HONDA et al. 1997; HONDA and YASUDA 2000). The biological relevance of MDM2 in the p53 pathway became more evident through the light of in vivo experiments where embryonic lethality of Mdm2 deficient mice was rescued by deletion of p53 (JONES et al. 1995; MONTES DE OCA LUNA et al. 1995). This finding suggests that the embryonic lethality observed in mice was a result of p53 activation due to the lack of negative regulation by Mdm2.

MDM2 is a 491 aminoacid protein that consists of an N-terminal p53 binding domain, import and export signals, an acidic domain, a zinc finger motif and a C-terminal RING domain. Both, the N- terminal and C- terminal domain of MDM2 play critical roles in the negative regulation exerted on p53. The N-terminal p53 binding domain directly interacts with the tumor suppressor transactivation site (CHEN et al. 1993). This interaction antagonizes p53 ability to carry out transcriptional activation of downstream gene targets that are involved in cell cycle control. Additionally, the C-terminal RING domain confers the E3 ubiquitin ligase activity through which MDM2 can target p53 for nuclear export and proteosomal degradation (HONDA et al. 1997). Interestingly, MDM2 is also subjected to negative regulation through the binding interaction with the tumor suppressor ARF (POMERANTZ et al. 1998; STOTT et al. 1998). The MDM2-ARF protein interaction mediated by the acidic domain results in the nucleolar localization of MDM2 and consequently in the inhibition of the negative regulation on p53 (WEBER et al. 2000). This interaction is required to protect the cells from
the oncogenic insult that results from abnormal mitogenic signals induced by overexpression of oncoproteins such as Myc, E1A, E2F1, Ras and v-Abl (BATES et al. 1998; DE STANCHINA et al. 1998; PALMERO et al. 1998; RADFAR et al. 1998; ZINDY et al. 1998). Once the oncogenic insult has been “sensed” by ARF, p53 dependent growth arrest or apoptosis is induced by the MDM2-ARF synergism. Oppositely, p53 activation can also occur as a result of DNA damage induced stress, which disturbs the MDM2-p53 interaction. In this alternative mechanism, homeostatic conditions favor the MDM2-p53 complex where the tumor suppressor is exported to the cytoplasm and degraded by the 26S proteasome. In this way the levels of p53 are kept low when there is not a requirement for cell cycle arrest or apoptosis. However, in response to DNA damage induced stress, the MDM2-p53 interaction is destabilized by the action of stress induced post transcriptional modifiers that will target both proteins in order to mediate a p53 dependent cell protection response (APPELLA and ANDERSON 2001; BROOKS and GU 2003; PRIVES and HALL 1999). Because MDM2 is under the transcriptional control of p53, the MDM2 transcript is also increased with the tumor suppressor activation. Consequently, once the stress signals that initiated the protection response disappear, the newly synthesized MDM2 promotes the reestablishment of p53 low levels by interacting again with the tumor suppressor (Figure 5) (LEV BAR-OR et al. 2000; STOMMEL and WAHL 2005). Therefore the MDM2-p53 interaction is characteristic of a negative feedback loop by which p53 levels are regulated either under homeostatic or DNA damage induced stress conditions. Overall, MDM2’s sensitivity to cellular signals from both misregulated
oncogenes and DNA damage makes it a critical regulator of the protective p53 response.

1.6 Alternative splicing of MDM2 and cancer

Inactivation of the tumor suppressor p53 is an event by which tumorigenesis can be initiated and association with overexpressed MDM2 provides a mechanism that leads to this event. Supporting this, MDM2 has been shown to be upregulated by means of gene amplification in 7% of all solid tumors and in 30% of soft tissue sarcomas (MOMAND et al. 1998). However, Bueso-Ramos et al., showed MDM2 overexpression in breast carcinomas and leukemias, with detection of several protein isoforms, is not dependent on MDM2 gene amplification but related to overexpression of MDM2 mRNA (BUESO-RAMOS et al. 1995; BUESO-RAMOS et al. 1996; BUESO-RAMOS et al. 1993). They also observed that the MDM2 mRNA overexpression identified in breast carcinomas and leukemias was comparable to the one detected in sarcomas where the MDM2 gene is amplified. This not only made clear that a mechanism other than gene amplification could drive MDM2 overexpression in tumors but that the pathway to MDM2 overexpression is tissue specific. The first human tumor related alternative spliced MDM2 transcripts were identified by Sigalas et al., in 1996 (SIGALAS et al. 1996). They detected six distinct MDM2 variants including full length, in ovarian and bladder tumors and in leukemia cell lines whereas only full length MDM2 was detected in six normal tissues and four benign ovarian
tumors. Interestingly, four of the five alternatively spliced forms lacked the p53 binding domain. Even more, the transforming ability of each of these spliced isoforms was shown by their independent transfection in NIH3T3 cells and their ability to generate transformed foci at high frequency (SIGALAS et al. 1996). Since then, these and other MDM2 splice variants have been identified also in soft tissue sarcomas, rhabdomyosarcoma tumors and cell lines, glioblastomas, liposarcomas and lung carcinomas (BARTHEL et al. 2001; EVDOKIOU et al. 2001; LUUKAS et al. 2001; MATSUMOTO et al. 1998; SIGALAS et al. 1996; TAMBORINI et al. 2001). However, the specific role of these spliced forms in tumorigenesis is not yet fully elucidated.

The MDM2 transcript can be generated from two independent promoters, P1 or P2. Eventhough transcripts generated from the P1 and P2 promoter lack exon 2 and exon 1 correspondingly, the resultant protein for both promoters is identical since translation of the transcript begins at exon 3 (BROWN et al. 1999; ZAUBERMAN et al. 1995). However, one important feature about the transcription of MDM2 from these two promoters is that, P2 transcription is p53 sensitive and 6-fold greater than transcription from P1. Moreover, a T to G single nucleotide polymorphism (SNP309G) at P2 can stimulate the transcription of MDM2 at this site through the transcriptional control of Sp1 (POST et al. 2010). The full length MDM2 protein results from a spliced transcript that contains exons 3 to 12, and is completely capable of inhibiting p53 by association and/or ubiquitination (Fig. 6). In contrast, MDM2-ALT1, the most common alternative spliced form of MDM2 detected in a variety of tumors, lacks internal exons 4-11, and results in a protein
isoform that is deficient for p53 interaction due to the lack of the p53 binding domain (Fig. 6) (Barcelo et al. 2001; Evdokiou et al. 2001; Lukas et al. 2001; Matsumoto et al. 1998; Sigalas et al. 1996; Tamborini et al. 2001). MDM2-ALT1 is induced in response to DNA damage stress and the protein product can associate with MDM2 full length to drive its localization to the cytoplasm. This MDM2-MDM2-ALT1 association results in increased activity of p53 and expression of its downstream gene targets (Chandler et al. non published data) (Fig. 6) (Chandler et al. 2006a; Evans et al. 2001). This negative regulation of MDM2-ALT1 on full length MDM2 is inconsistent with the emergence of this alternative spliced isoform in human tumors and its described transformation potential in NIH3T3 cells.

MDM2 can promote cell proliferation through its interaction with p53 but also stimulate cell arrest through its interaction with ARF and MDM2-ALT1. In normal cells, MDM2 interaction with ARF and MDM2-ALT is part of a protective response when cells are being submitted to oncogenic insult or DNA damage response correspondingly. In this case, p53 levels are upregulated, resulting in cell cycle arrest that will either be resumed to normal after the insult or stress is no longer present or terminated by driving cells that have been irreversibly damaged to undergo apoptosis. Based on the current known role of MDM2-ALT1 it is not understood how its expression can co relate with the emergence of tumors. Previous findings regarding the tumorigenic role of the alternative spliced forms of MDM2 suggest that that their mechanism is dependent on the cellular context in which they are expressed and moreover in the genetic background of
the cells. Therefore, an in vivo model system to elucidate how the MDM2’s spliced variants can contribute to malignancy is a promising tool to clarify the still unanswered questions regarding their tumorigenic potential.

In this study, we have established a DNA damage inducible alternative splicing system in which cells recapitulate the alternative splicing events that give rise to the observed expression of MDM2-ALT1 in cancer. Using this system we have taken a mechanistic approach to discover the regulation behind the alternative splicing of MDM2. Moreover, we have developed an MDM2-ALT1 transgenic mouse model to study the expression of this MDM2 isoform in a relevant in vivo model where the findings can have direct implications in the appearance of B cell related tumors.
Figure 1. Eukaryotic gene expression. Genes undergo transcription to generate a pre-mRNA. This pre-mRNA undergoes the post-transcriptional modifications of capping, polyadenylation and splicing to yield a mature mRNA that can be effectively transported to the cytoplasm for translation into a protein.
Figure 2. Cis-acting elements of pre-mRNA splicing: Core splicing signals and regulatory splicing signals. A. The core splicing signals consist of the GU dinucleotide at the 5’ splice site, the AG dinucleotide at the 3’ splice site and the branch point sequence, containing the particular adenine nucleotide followed by a polypyrimidine tract. These signals, though nearly invariant, are degenerate and therefore by itself are not enough to precisely recruit the spliceosome machinery to the correct splicing sites. B. Cis-acting regulatory sequences called enhancers and silencers, laying at the exons and introns, promote the binding of splicing factors at these sites as a mean to accurately define the exon/intron boundaries. Therefore the splicing machinery, through interaction with these splicing factors is recruited to accurate splicing sites.
Figure 3. The biochemical reaction of pre-mRNA splicing. In the first step of splicing the hydroxyl group of an adenine residue at the branch point sequence attacks the phosphate at the 5’ splice site. This results in the excision of the 5’ exon from the intron and in the joining of the intron 5’ to the branch point 2’ hydroxyl. In the second step, 3’ hydroxyl group of the excised exon attacks the phosphate at the 3’ end of the intron. This reaction ligates the two exons and releases the intron in a lariat configuration.
Figure 4. Spliceosome assembly. The spliceosome assembles in the pre-mRNA in a stepwise fashion where initially the U1 snRNP binds the 5' splice site, U2AF65 binds the 3' splice site, SF1/BBP binds the branch point and U2AF65 binds the branch point sequence. These interactions give rise to the ATP independent formation of the E complex. In the A complex, SF1/BBP is displaced from the pre-mRNA while U2 takes its place at the branch point sequence in an ATP dependent reaction. U4, U5 and U6 get recruited to the pre-mRNA as a pre-assembled and inactive B complex. The A complex associates with the B complex through an interaction between the U2 and U5 snRNP, which results in the displacement of U1, U4, U2AF35 and U2AF65 and in the activation of the B complex. Once U1 and U4 are displaced the active C complex catalyzes the first ATP dependent splicing reaction. After rearrangement of the snRNP complexes, the second ATP splicing reaction takes place followed by disassembly of the spliceosome and release of the fully spliced mRNA product and the intron in a lariat configuration.
Figure 5. The MDM2-p53 pathway. Under homeostatic conditions MDM2 inhibits p53 by binding its trans activation domain and by targeting it for proteosome degradation. However, upon cellular stress conditions the MDM2-p53 interaction is disrupted through the post translational modification of both proteins. This results in the stabilization of p53 and in the transcriptional activation of its downstream targets. If cells are able to revert the damaging event that triggered this protective response, cell cycle is resume. Otherwise, the apoptotic cell death program is initiated. Because MDM2 is also under the transcriptional control of p53, once the cell fate is determined, the MDM2-interaction is re established and p53 levels return to normal.
Figure 6. Regulation of p53 through alternative splicing of MDM2. The MDM2 pre-mRNA consists of exons 3 to 12. Upon homeostatic conditions the MDM2 transcript undergoes constitutive splicing of its exons giving rise to an mRNA that upon translation yields the full length MDM2 protein. Full length MDM2 is capable of interacting with the tumor suppressor p53 and of targeting it for degradation. However in response to stress and in cancer the MDM2 transcript undergoes drastic alternative splicing where its internal exons are excluded to drive the expression of MDM2-ALT1, a protein isoform that lacks the p53 binding domain, and stabilizes p53 through an interaction with full length MDM2.
Chapter 2: Regulatory interplay between *cis*-elements in exon 11 and *trans*-factors coordinate the constitutive and alternative splicing of *MDM2*.

2.1 Introduction

In higher eukaryotes, proteome diversity and complexity is enhanced by the powerful mechanism of alternative splicing. Alternative splicing is the major source of gene expression plasticity since using the same gene template, a protein and its isoforms can be generated upon the requirement of the specific cellular process that needs to be accomplished. Because failure to generate properly spliced transcripts can result in misregulated, unrequired and non functional proteins that can have a negative effect in cell homeostasis, both constitutive and alternative splicing need to take place under highly regulated mechanisms. Therefore, constitutive and alternative splicing need to be stringent enough to maintain efficient regulation but still be dynamic to suffice the many changing metabolic requirements of a cell.

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This is achieved through the interplay between regulatory sequences embedded in the pre-mRNA and splicing factors that are sensitive to different cellular stimulus.

SF2/ASF and SC35 are two splicing factors that belong to the SR protein family of phosphoproteins and they are required for the first step of splicing and for assembly of the spliceosomal A complex (Fu and Maniatis 1990; Fu and Maniatis 1992; Ge and Manley 1990; Kraliner et al. 1990; Mayeda et al. 1992; Roth et al. 1991; Spector et al. 1991; Zahlen et al. 1992). SF2/ASF is a key player in activation of 5' splice site. SC35 can also regulate the activation of 5' splice site in addition of promoting the interaction between U1 and U2 snRNP’s (Zuo and Manley 1993; Zuo and Manley 1994). The regulation of 5' splice selection by both SF2/ASF and SC35 has been shown to be concentration dependent in in vitro experiments with duplicated 5’ splice sites, where the usage of the 5’ proximal splice site is favored upon increasing levels of any of this two splicing factors. Interestingly, the 5’ splice site selection activity of SF2/ASF and SC35 is antagonized in a concentration dependent fashion by the silencing factor hnRNP A1. The 3’ splice selection is also regulated by these splicing factors in a dosage dependent manner, but hnRNP A1 does not interfere with this modulation (Fu et al. 1992; Mayeda and Kraliner 1992). In vivo, changes in the expression pattern of these proteins can occur as to satisfy the temporal, developmental or tissue specific requirement or as a result of a cellular stimulus response. In line with this, Singh et al. (data not published) has observed that
upon induction of DNA damage stress, MCF7 cells show redistribution of the
SC35 splicing factor to nuclear speckles, in what might be a cellular protective
response. Unfortunately, the same mechanisms by which splicing factors can be
regulated to favor cellular homeostasis can lead to their misregulation and have a
negative impact for cells. Accordingly, cumulative evidence has emerged linking
the increased expression of SR proteins to cancer. The SR protein Srp20 is
elevated during late stages of a mammary tumorigenesis model, SRp20,
SF2/ASF and SC35 are overexpressed in ovarian cancer and SF2/ASF has been
categorized as a proto oncogene due to his transforming ability in mouse
fibroblasts when overexpressed (FISCHER et al. 2004; HE et al. 2004; KARNI et al.
2008). Overall, the exclusion/inclusion of an exon through the exon/intron
definition process is the result of a myriad of protein:protein / RNA:protein
interactions that rely on exon length, integrity of regulatory sequences and
regulated expression of splicing factors to determine the final outcome of the
spliced pre-mRNA.

This study, aims to identify the regulatory elements that govern the
alternative splicing of the MDM2 gene transcript. It has been reported that
alternative splicing of MDM2 is induced in response to specific DNA damage
stress and is conserved between human and mouse (CHANDLER et al. 2006b).
This induction allows for the detection of different MDM2 spliced forms, MDM2-
ALT1 being the most predominant (CHANDLER et al. 2006a). A minigene is a
smaller version of a gene that contains the alternative exon (s), the surrounding
introns and the flanking constitutive exons that are to be analyzed in the identification of the features that control their usage (Stoss et al. 1999). This genomic fragment is cloned into an eukaryotic expression vector and used in transient transfections to identify cis-acting elements and trans-acting factors that regulate constitutive and alternative splicing (Cooper 2005). Our group has generated a splicing competent MDM2 minigene system that upon transfection of human cell lines and induction of stress gives rise to the detection of MDM2-ALT1, the most common spliced form of MDM2 detected in tumors. We used this stress inducible alternative splicing system that parallels the splicing of MDM2 observed in cancer as to understand and identify the events that drive the expression of MDM2-ALT1 in tumors. We have generated an array of minigene constructs where predicted ESE sequences have been altered as to decrease the binding affinity of SC35 and SF2ASF. Finally, using these minigenes in transient transfection splicing assays we showed that ESE sequences located in exon 11 are important for both constitutive splicing of full length MDM2 and also for damage induced alternative splicing of the MDM2 transcript.

2.2 Materials and Methods

Cell Culture. MCF7 (human breast carcinoma cell line, p53 wild type), H1299 (human lung cancer cell line, p53 null) and Hela (human cervical carcinoma cell p53 wild type) cell lines were maintained in DMEM supplemented with 10% fetal
bovine serum (HyClone, Logan, UT), 2mmol/L L-glutamine, and 0.1 mg/ml penicillin (100IU/mL) / streptomycin (100 ug/mL) at 37°C in a humidified atmosphere of 5% CO2.

**Construction of MDM2 minigenes.** The 3-4-10-11-12, 3-10-11-12 and 3-11-12 minigenes were generated as previously described (SINGH et al. 2009). The 3-11-12 406 minigene was engineered by deleting the majority of intron 11 in the 3-11-12 minigene (Singh et al., unpublished data). Briefly, this was achieved by amplification and cloning of a smaller intron 11 and exon 12 into the EcoR1 and Xho1 sites in the 3-11-12 minigene using Int 11 del 4 ER1 F as the forward primer and X12 HDM2Xh1 R as the reverse primer. Partial deletion of exon 12 was carried out by cleavage and re ligation using the restriction enzyme Apa 1 site in the pCMV-tag 2B’s multiple cloning site and also in exon 12 of MDM2. Additionally, the 5’ site of exon 3 was deleted by PCR amplification and cloning into Bam H1 and Pst 1 restriction enzyme sites using X3 del 1 BH1 F as the forward primer and X3 del 1 Pst R as the reverse primer. Also part intron 10 was deleted in this minigene by amplification and cloning exon 11 with smaller upstream intron 10 and downstream intron 11 into the Pst 1 and Eco R1 restriction enzyme sites using I 10 del 1 Pst F as the forward primer and X11 HDM2ER1 R as the reverse primer. The MDM2 3-11-12 406 mutant minigenes with disrupted ESEs were developed through analysis of the minigene using ESEfinder 2.0 (Cold Spring Harbor Laboratory) and by in vitro site directed mutagenesis using Quickchange II XL mutagenesis kit, (Stratagene, La Jolla,
CA). A description of the primers used to engineer the MDM2 3-11-12 406 minigene and the minigenes with the disrupted ESEs appears in table 1.

**Evaluation of minigenes using transient transfection splicing assays.** Each minigene was individually transfected into MCF7, H1299 and Hela cell lines using Fugene 6 (Roche Diagnostics Corporation, Indianapolis, IN). Cells were exposed to UVC (50J/m²) 20 hours post transfection. Total RNA was harvested 24 hours after damage treatment with UVC using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was generated using Transcriptor reverse transcriptase (Roche Diagnostics, Indianapolis, IN) with random hexamer primers. To evaluate the alternative splicing of the indicated minigenes, the cDNAs were submitted to PCR using the FLAG tag sequence as the forward primer and the exon 12 sequence, 5’-CAATCAGGAACATCAAGCC-3 as the reverse primer. This primer set restricts the PCR amplification to transfected MDM2. PCR products were visualized on an electrophoresed 2% agarose gel. Quantification of bands corresponding to spliced products was carried out using ImageQuant 5.2 software (GE Healthcare Life Sciences).

**2.3 Results**

**Splicing competent MDM2 minigenes as a biochemical tool to study damage induced splicing in vivo.** As it was described in chapter one, splicing and alternative splicing are part of a very complex network of interactions, where different layers of regulation assure that these processes are achieved accurately and precisely. An effective study and understanding of the splicing regulation in vivo can be achieved through the usage of a minigene, which simplifies the
analysis by limiting the length of the genomic fragment under study (COOPER 2005; STOSS et al. 1999). Therefore, our group generated two initial minigenes that contain exon 3 and exon 12 and the corresponding surrounding exonic and intronic sequences. These two minigenes are the 3-10-11-12 minigene and the 3-4-10-11-12 minigene (Fig. 7A) and both were engineered by PCR amplification and cloning of the included genomic sequences of MDM2 in a pCMV-tag2B vector. To test the efficiency of these minigenes to undergo DNA damage induced alternative splicing as observed endogenously (CHANDLER et al. 2006a), we carried out transient transfection of the individual minigenes in MCF7, H1299 and HeLa cell lines. After 24 hours of the cells being transfected, we induced genotoxic stress by treatment of the cells with 50 J/m² UVC. Total RNA was harvested 20 hours post-treatment and submitted to RT-PCR. We took a PCR amplification approach that is specific for the detection of the minigene transcripts by using primers specific for the FLAG tag sequence in the minigene. We observed that for the three cell lines, in the absence of genotoxic stress, the main spliced form generated from the 3-4-10-11-12 minigene contained all of the internal exons between exons 3 and 12 (Fig 7B, lanes 8, 12, 18). However, transfected cells that were treated with UVC, showed skipping of the internal exons 4 to 11 resulting in the induction of MDM2-ALT1 (Fig. 7B, lanes 5, 11, 17). Moreover, this MDM2-ALT1 induction is increased in all of the treated cell lines in comparison with the cell lines that were not submitted to the genotoxic insult. Accordingly, when analyzing the 3-10-11-12 minigene, in the absence of DNA
damage, inclusion of the internal exons between exon 3 and 12 is mainly observed in the three cell lines under study (Fig. 7B, lanes 4, 10, 16), whereas exclusion of the internal exons and the yield of \( MDM2-ALT1 \) is recognizable upon treatment with UVC (Fig. 7B, lanes 3, 9, 15). The induction of \( MDM2-ALT1 \) from the 3-10-11-12 minigene, upon stress conditions in the three cell lines is also enhanced in comparison to non treated cells.

Our lab has previously shown that \( MDM2-ALT1 \) can also be induced endogenously, through the induction of similar stress by treatment of the cells with Cisplatin, a chemotherapeutic drug that promotes DNA crosslinking with platinum complexes (CHANDLER et al. 2006a). Therefore, we repeated the above described experiments, using Cisplatin as the DNA damaging agent through which cellular stress would be exerted. Also in this experiment, the cells that were submitted to DNA damage stress, this time by Cisplatin treatment, showed enhanced levels of the \( MDM2-ALT1 \) form upon transfection of the 3-4-10-11-12 or 3-10-11-12 minigene in H1299, MCF7 and HeLa cell lines (Fig. 7C, lanes, 3, 5, 9, 11, 15, 17), whereas the main form generated in untreated cells includes all the internal exons (Fig. 7C, lanes 4,6,10,12,16,18). As a result of these experiments, we conclude that the 3-4-10-11-12 and 3-10-11-12 minigenes are stress induced splicing competent and that they therefore contain the \( cis \)-acting elements that regulate the damage induced alternative splicing of \( MDM2 \) observed endogenously. We have also established that our PCR approach is effective in the restricted detection of spliced forms arising from the transfected
minigenes. The final outcome of these experiments is the establishment of a minigene system that resembles the splicing of *MDM2* upon conditions of stress as a valuable tool to study its splicing regulation and the implications of this process in cancer.

**Conserved ESE’s in exon 11 of MDM2 regulate its constitutive and damage induced alternative splicing.** A comparison between the human and mouse genome showed the conservation of intronic sequences and suggests that the role of these sequences is the regulation of alternative splicing in both species (SOREK and AST 2003). Accordingly, our group has demonstrated that damage induced alternative splicing of *MDM2* is conserved between human and mouse (CHANDLER et al. 2006b). Our lab carried out a comparative analysis between the genomic sequences in the 3-10-11-12 MDM2 minigene and mouse *Mdm2* gene. We have shown in *in vitro* splicing assays using a 3-11-12 minigene where intron 11 only contains significantly conserved nucleotides between human and mouse, that conserved sequences within the *MDM2* intron 11 regulate the observed differential usage of exon 11 in response to damage (SINGH et al. submitted). Because we wanted to identify ESE’s that could regulate the splicing of *MDM2*, we engineered a smaller MDM2 minigene, 3-11-12 406 minigene (Fig. 8A), where the 3’ splice site and the polypyrimidine tract of intron 11 is kept but most of the conserved intronic region is deleted as a means to limit the splicing regulation to the role of not yet discovered exonic cis-elements. Using the online prediction program of ESE values, ESEfinder 2.0, we
query the genomic sequences of MDM2 in our 3-11-12 406 minigene. Sequence comparison between human and mouse MDM2 genes resulted in the identification of conserved ESE’s in exons 3, 11, 12 that predict the binding of the SR protein SC35 (Fig. 8A, green bars). In the same way, conserved ESEs for the SR protein SF2/ASF were identified in exons 3 and 11 (Fig. 10A, yellow bars). Even more, thorough analysis of our MDM2 406 minigene sequences through ESEfinder, led us to identified two overlapping SF2/ASF ESEs at exon 11. These ESE’s share a common central region but have differential starting and ending dinucleotides (Fig. 10B). We wanted to test the possibility that these newly found ESEs could have a functional role in the splicing regulation of MDM2. Therefore we carried in vitro site directed mutagenesis to generate 3-11-12 406 minigenes that would carry individual disruption of each of the predicted binding sites of these SR proteins. The mutated minigenes are identified based on the position of the ESE in the 3-11-12 406 minigene where 57, 73, 165, 213 and 382 correspond to ESEs promoting the binding of SC35 at those positions. We have outlined the exonic conserved sequences and their corresponding ESE values pre and post site directed mutagenesis (Fig. 8B). We then analyzed the effect of disrupting these ESE’s by individual transfection of the mutated minigenes in MCF7 cells and performing the in vivo splicing assay described in the previous section. In vivo splicing assay upon transfection of the 3-11-12 406 minigene in MCF7 as a control, showed that this minigene retained the required cis-elements for splicing regulation under normal and damage conditions (Fig. 9, A-B).
Individual transfection of the 3-11-12 406 minigenes with mutated SC35’s ESE 57, 73 and 382 showed a splicing pattern of the minigene transcript that is comparable to the 3-11-12 406 control on the in vivo splicing assay (Fig. 9A). Therefore, disruption of these SC35 ESEs did not affect the splicing pattern of the 3-11-12 406 minigene. However, individual transfection of MCF7 cells with the 3-11-12 406 minigenes with mutated SC35’s ESE 165 and 213 resulted in an increase of the MDM2-ALT1 form in which exon 11 is excluded. This increase in the exclusion of exon 11 is evident both in cells untreated and treated with UVC (Fig. 9A). Because the disruption of SC35 ESE’s 165 and 213 under normal and stress conditions (UVC treatment) results in a change in the splicing pattern that favors the expression of the MDM2-ALT1 form in which exon 11 is excluded our data suggests that these ESEs regulate both constitutive splicing under normal conditions and alternative splicing upon induction of genotoxic stress. Moreover, because these two ESE’s are predicted binding sites for SC35, and the interaction with SC35 is predicted to be disrupted upon the described mutations, our data suggests that this splicing factor is required to regulate the inclusion of exon 11 as part of the the splicing mechanism of MDM2.

In order to validate the findings of the previous experiment and to further confirm the suggested role of both SC35 and ESEs 165 and 213 in the splicing regulation of MDM2, we carried out compensatory mutations at these ESEs. In this approach, the aim is to revert the changes in the splicing pattern by introducing a new mutation that can restore the predicted binding affinity for an
SR protein, while keeping the initial mutation that disrupted the interaction. Therefore, we used ESEfinder 2.0, to determine the changes in the SC35 and SF2/ASF ESE’s that would restore the ESE values and consequently the binding of the SR proteins at those sites. Moreover, also using ESEfinder 2.0 we analyzed the compensatory mutations to assure that no other potential ESE is affected with the introduction of this change. We carried out compensatory in vitro site directed mutagenesis to introduce the new changes in SC35 ESE 165 and 213. The compensatory mutations in SC35 ESE 165 and 213 have been identified as comp 165 and comp 213 correspondingly and their description and predicted ESE values have been outlined (Fig. 8 C). We observed a moderate to slight increase in the inclusion of exon 11 upon analysis of the SC35 ESE comp165 and comp 213 by transfection of MCF cells untreated and treated with UVC (Fig. 9 B). The findings from the analysis of the SC35 ESE comp 165 and 213 validate the previous experiment and altogether suggest that ESEs 165 and 213 regulate the splicing of MDM2 through an interaction with SC35, that upon induction of stress is disrupted and results in the generation of the MDM2-ALT1 alternative spliced form.

ESEs 48 and 175 are predicted to promote the binding of SF2/ASF at these positions in the 3-11-12 406 minigene. The SF2/ASF ESE 175 contains overlapping ESEs and we have identified those as site #1 and site #2 (Fig. 10B). The exonic conserved sequences and their corresponding ESE values pre and post site directed mutagenesis are outlined (Fig. 10C). We similarly investigated
the role of SF2/ASF and ESEs 48 and 175 in the regulation of *MDM2* splicing. A change in the splicing pattern in comparison with the control minigene was not observed when the cells where transfected with the 3-11-12 406 minigene with mutated SF2/ASF’s ESE 48, indicating that this ESE is not necessary to regulate the splicing of the MDM2 minigene (Fig. 11A). Interestingly, transfection of the 3-11-12 406 minigene with mutated SF2/ASF’s ESE 175, showed an enhanced inclusion of exon 11, in cells untreated and treated with UVC, that is evident by the drastic decrease in the *MDM2-ALT1* form which appears undetectable in both conditions (Fig. 11A). This experiment shows that ESE175 can also regulate constitutive splicing under normal conditions and alternative splicing upon induction of genotoxic stress. Because the disruption of SF2/ASF ESE 175 under normal and stress conditions (UVC treatment) results in a change in the splicing pattern that favors the expression of the 3-11-12 form in which exon 11 is included our data suggests that SF2/ASF’s interaction with this ESE regulates the exclusion of exon 11. Most importantly, our results suggest a novel role for this SR protein as an exonic splicing silencer that promotes the exclusion of exon 11 in the splicing regulation of *MDM2*.

Again, we wanted to validate our observations regarding SF2/ASF and ESE175 in the splicing of MDM2. However, this time engineering an SF2ASF ESE 175 compensatory mutation represented more of a challenge. Our ESE finder analysis of the 175 ESE resulted in only one possible nucleotide combination that could re establish the original ESE value as to compensate for
the initial disrupting mutation. However, our only possible compensated sequence for ESE 175, would only re establish the ESE value for a predicted SF2/ASF interaction at ESE 175 site #2 (Fig. 10B, D). Transfection of the SF2/ASF ESE comp 175 in MCF7 cells untreated and treated with UVC, proved to not be sufficient to rescue the splicing pattern under both normal and damaging conditions. Furthermore, we observed that inclusion of exon 11 is even more dramatic in the cells transfected with the ESE comp 175 in comparison with ESE 175. Because the ESE comp 175 restricts the predicted ESE value re establishment only for site #2, we could not assess if restoration of ESE values for ESE 175 site #1 could have been more effective in rescuing the splicing pattern of the SF2/ASF ESE 175 minigene. Also, because the ESE 175 mutation disrupts both sites #1 and #2, we could not tell what could be the independent role of these two overlapping ESEs. We therefore decided to first determine if disruption of both SF2/ASF ESE 175 site #1 and site #2 is required for the observed inclusion of exon 11. To do this we engineered mutated ESE 175 sequences that would lower the ESE value at site #1 and site #2 independently and consequently disrupt the predicted SF2/ASF interaction at one ESE site but not the other one. We have identified these newly mutated ESEs as SF2ASF ESE 175 S1, S2 and S1+S2 and a description of these sequences including the change in ESE values has been outlined (Fig. 12 A). Only MCF7 cells transfected with a minigene mutated at the ESE 175 S1, showed the drastic inclusion of exon 11, under both normal and UVC damaging conditions (Fig. 12B,
lanes 7-8, 11-12). However, MCF7 cells transfected with the minigene mutated at
the ESE 175 S2 showed a splicing pattern that resembles the one observed
using the 3-11-12 406 minigene as a control, suggesting that disruption of this
ESE site is not enough to affect the splicing regulation of MDM2 under the
studied conditions (Fig. 12A). We wanted to further validate our finding regarding
SF2/ASF ESE 175 site #1 by performing a compensation experiment. However,
no nucleotide alteration in the mutated ESE 175 S1 would restore the predicted
ESE value while keeping the original disruption mutation. We therefore
engineered a second ESE 175 site #1 mutation that would both disrupt the ESE
but allow for compensation. Unfortunately, the only possible sequence alteration,
the ESE 175 S1#2, which would allow for compensation, also enhances ESE 175
site #2. The SF2/ASF ESE 175 S1#2 sequence alteration and its predicted ESE
value is described in figure 13A. We also outlined the sequence for the pertaining
compensatory mutation identified as SF2/ASF ESE 175 comp S1#2 and its
predicted ESE value (Fig. 13B). Analysis of these mutated ESE showed the
same previous results. The SF2/ASF ESE 175 S1 #2 effectively promoted the
inclusion of exon 11 possibly by disrupting the predicted SF2/ASF interaction at
ESE 175 site #1 but the compensatory ESE 175 comp S1 #2 sequence failed to
rescue the splicing pattern (Fig. 13C). Therefore, our observation regarding the
role of ESE 175 and SF2/ASF in the splicing regulation of MDM2 remains to be
further validated.
2.4 Discussion

Eukaryotic pre-mRNA splicing involves the removal of introns and the joining of exons to yield a translatable mature mRNA. Branching out from splicing is alternative splicing, a highly regulated process in which differential exons are included or excluded from the final mRNA product to generate different protein isoforms from the same genomic DNA template. Alternative splicing provides a mechanism that allows for the expression of a particular protein isoform upon the onset of the variant cellular requirements that need to be fulfilled to preserve homeostatic conditions. MDM2 is alternative spliced in response of stress and many alternative spliced forms have been detected in several tumors and cancer cell lines, being MDM2-ALT1 the predominant. Our group and others have shown that MDM2-ALT1 disturbs the MDM2-p53 interaction by sequestering full length MDM2 to the cytoplasm (Handler et al. 2006a; Evans et al. 2001). This results in the stabilization of p53 and in the transcriptional activation of its downstream gene targets. Eventhough, the alternative splicing of MDM2 evidently has relevant implications in cancer, not much is known about the events that drive the cell to initiate this process. Therefore, in this study we have undertaken a systematic approach to identify the regulatory mechanism behind the alternative splicing of MDM2 as a way to understand the events taking place in tumor cells that lead to the generation of these alternative spliced forms. We have developed an MDM2 minigene system that undergoes alternative splicing upon the induction of genotoxic stress (UVC or Cisplatin) and that allows for the
restricted detection of alternative spliced forms arising specifically from the transfected minigene. Most importantly, damage induced alternative splicing of our 3-10-11-12, 3-4-10-11-12, 3-11-12 and 3-11-12 406 in transient transfection of human cell lines results in the detection of MDM2-ALT1. Therefore, we have developed a damage induced alternative splicing system that resembles the alternative splicing of endogenous MDM2 observed in tumors and that consequently can be used as a model to identify the regulatory cis-elements and trans-factors that coordinate the splicing of MDM2 in cancer.

There is compelling scientific evidence that supports a p53 independent role for MDM2 in tumorigenesis. In line with this, it has been shown that Mdm2 ubiquitous overexpression could promote tumorigenesis in p53 null mice and that Mdm2 confers a cellular growth advantage that results in transformation of p53 null cells (DUBS-POTERSZMAN et al. 1995; JONES et al. 1998). Moreover, alternative spliced forms of MDM2 that are unable to bind p53 have been shown to induce tumorigenesis (FRIDMAN et al. 2003; STEINMAN et al. 2004). Our initial experiments with the 3-10-11-12 and 3-4-10-11-12 minigenes also show that alternative splicing of MDM2 takes place independent of p53 status, since H1299 cells, a p53 null cell line, were capable of undergoing alternative splicing of the transfected minigenes.
In our introduction chapter we discussed how splicing control is the result of additive regulation layers in which the interaction between cis-elements and trans-acting factors, for its critical role in promoting efficiency, accuracy and fidelity of the process, emerges as a crucial element in the modulation and in the final outcome of splicing. We have used our MDM2 3-11-12 406 minigene to further prove the relevance of the coordinated action of cis-elements and trans-factors in the splicing regulation of MDM2. We showed that conserved ESEs in exon 11 of our MDM2 3-11-12 406 minigene have a regulatory role in splicing of MDM2 under normal and damaging conditions and that if disrupted could alter the splicing pattern of this transcript. Moreover, because these ESEs are predicted to bind specific SR proteins, one can also assess the role of these trans-factors in splicing through the inhibition of their interaction with the ESE under study. ESEfinder, an online prediction program of exonic splicing enhancer sequences, provides values for a predicted interaction between an exonic sequence and an SR protein. We show that our 3-11-12 406 minigene contain conserved ESE sequences at exons 3, 11 and 12 that are predicted to promote an interaction either with the SR protein SC35 or SF2/ASF. In vitro site directed mutagenesis of these ESEs showed that SC35 ESEs 165 and 213 and SF2/ASF ESE 175 have a regulatory role in the splicing of MDM2. A G→T nucleotide substitution in SC35 ESEs 165 and 213, results in a lower ESE value for the interaction of SC35 with these altered exonic sequences. Consequently, this G→T substitution at ESE 165 and 213 resulted in an increase
in the exclusion of exon 11 that is evident by the enhanced detection of the MDM2-ALT1 fragment in cells that were transfected with the mutated ESEs minigenes in comparison to cells transfected with the 406 minigene as a control. Interestingly, the increase in the exclusion of exon 11 was detected in cells that were untreated and treated with UVC. This suggests that SC35 ESEs 165 and 213 have a regulatory role in constitutive splicing under normal conditions but also can regulate alternative splicing of MDM2 under DNA damaging conditions. Moreover, because SC35 is predicted to bind to these ESEs and its interaction with these sequences is predicted to be inhibited by the nucleotide substitutions, then SC35 loss of function can also be associated with the observed increase in exon 11 exclusion. Based on these findings we therefore propose that SC35 plays a role as a positive regulator of MDM2 splicing to promote inclusion of exon 11 under both normal and damaging conditions.

The proposed role of SC35 and ESEs 165 and 213 in the splicing of MDM2 was further validated. This was achieved by analysis of MCF7 cells transfected with the MDM2 3-11-12 406 minigenes in which a compensatory mutation was introduced in the mutated ESE as a mean to compensate for the original disruption and to rescue the splicing of the minigene. The compensatory mutations for the mutated ESE 165 and 213 resulted in a higher ESE value for the predicted interaction of SC35 with these ESEs and in a moderate to slight increase in the inclusion of exon 11. In the introduction to this chapter we highlighted how ESE’s and ISE’s can promote differential splice site usage.
depending on the binding affinity of an SR protein on a particular sequence. According to ESEfinder, for optimal interaction with SC35, the last nucleotide of an ESE sequence is likely to be an A or a G, as it is the case of the unmutated ESE 165 and 213. A possible explanation for the observed subtle rescue in splicing, might be that the compensatory mutations introduced, even though restoring the ESE values, were not enough as to suffice the specific requirement of an A or a G at this particular position in the sequence. It would be interesting to see if a disrupting mutation that does not compromise this particular nucleotide, would have similar splicing effects but moreover could be better compensated. In any case, such experiment, if successful, would further confirm the already well established idea that differences in SR protein binding affinities for ESEs, serve as a mechanism that regulates differential splicing. In the meantime, our findings from the compensatory experiments further confirm that both ESEs 165 and 213 as cis-acting elements and SC35 as a trans-acting factor, can positively regulate the splicing of \textit{MDM2} by promoting the inclusion of exon 11.

A thorough analysis of our MDM2 3-11-12 406 minigene using ESE finder led to the discovery of two predicted sites within ESE 175 that could promote an interaction with the SR protein SF2/ASF. A C→A substitution in the third position within the overlapping sequence of these two sites, lowers the predicted ESE values for an interaction with SF2/ASF at both site #1 and #2. Cells transfected with this mutated ESE minigene showed drastic inclusion of exon 11 to the extent
that the **MDM2-ALT1** fragment is almost undetectable. Also, the increase in exon 11 inclusion was evident under both normal and stress conditions. These results show that ESE 175, as a *cis*-acting element, can also regulate MDM2 splicing through an interaction with SF2/ASF. Moreover, the data shows that a predicted lower interaction between the mutated ESE 175 and SF2/ASF results in a change in the splicing pattern where exon 11 inclusion is enhanced. This suggests that, opposite to the proposed role of SC35 in MDM2 splicing, SF2/ASF can negatively regulate the splicing of MDM2, by promoting the exclusion of exon 11 under normal and stress conditions.

In our aim to validate our findings regarding SF2/ASF and ESE 175 through a compensatory mutation experiment, we found that ESE 175 site #1 was the exonic sequence promoting the previous observed inclusion of exon 11 under both normal and stress conditions. While a restricted disruption of ESE 175 site #1 was enough to detect the observed increase in the inclusion of exon 11, an independent disruption specific for ESE 175 site #2 failed to produce any changes in the splicing pattern of the mutated 3-11-12 406 minigene when compared with the control. This suggest that at least under the conditions studied, these two sites might have different regulatory roles, since the disruption of ESE 175 site #1 cannot be compensated by an intact ESE 175 site #2. Moreover, the finding that there are two distinct regulatory sites within SF2/ASF ESE 175 altogether with our finding of two other regulatory SC35 ESE also lying
within exon 11, shows the high level of regulation of this exon to undergo alternative splicing through the interplay of both \textit{cis}-elements and \textit{trans}-factors.

Although our compensatory experiments to re-establish splicing using the different mutated SF2/ASF ESE 175 minigenes failed to show results that could further validate our proposed role for SF2/ASF and this ESE in the splicing of MDM2, our findings about how disruption of this ESE impacts the splicing of the MDM2 3-11-12 406b minigene should not be disregarded. Evidently, the disruption of SF2/ASF ESE 175 abolished the alternative splicing of MDM2 where exon 11 is skipped in response to DNA damaging conditions. However, upon introduction of the compensatory mutations we observed an even higher increase in the inclusion of exon 11. It could be possible that disruption of SF2/ASF interaction with ESE 175 upon the nucleotide substitution permits the interaction of a silencing factor with the mutated ESE. In that case, a rescue in the splicing pattern would not be detectable unless the compensatory mutation is capable to disrupt the new interaction between the ESE and the silencer. The role of \textit{trans}-acting silencers to inhibit exclusion of an exon is consistent with our observed increase in exon 11 inclusion even after the introduction of a compensatory mutation.

The Human Splicing Finder (HSF) is an online tool that has integrated all available matrices to identify intronic and exonic motifs as well as new matrices for the identification of interactions with the silencer hnRNP A1 among other factors \cite{DESMET et al.}. We have queried the SF2/ASF ESE 175 sequence using
this online tool and consistent with the previously described idea, we discovered that this sequence resembles a silencer motif described by Sironi, M et al. in 2004 and it predicts that the ESE 175 sequence has an over the threshold value to function as a silencer sequence (Sironi et al. 2004). Moreover, a new experimental matrix to discover hnRNP A1 motifs, predicts that a partial sequence of the ESE 175 can mediate an interaction with this silencing factor and also gives the sequence an over the threshold value for the interaction (Desmet et al.). Overall, this finding further confirms that inclusion/exclusion of exon 11 is highly regulated by the interaction between regulatory sequences within the exon and splicing factors that can either promote or inhibit splicing.

A current study in our lab has shown, through an RNP pull down experiment that T7 tagged SC35 and SF2ASF transfected in MCF7 cells are capable to complex with endogenous MDM2 pre-mRNA (Singh and Chandler, unpublished data). We have also observed that overexpression of SC35 in MCF7 cells results in an increase in exon 11 inclusion under stress conditions whereas overexpression of SF2/ASF in the same cell line results in an increase in the exclusion of exon 11 under both normal and damaging conditions (Singh and Chandler, unpublished data). Most interestingly, we have also observed that in response to stress induced by UVC treatment, though the levels of SC35 remain the same, MCF7 cells show a redistribution of SC35 to form larger and fewer nuclear speckles. On the contrary, SF2/ASF, which does not appear to undergo the redistribution pattern observed by SC35 in response to UVC treatment, is
increased in Hela S3 cells that have been treated with Cisplatin, but most importantly is also increased in rhabdomyosarcoma patient samples (Singh and Chandler, unpublished data). Therefore, these observations together with the findings presented in this manuscript support the idea that SC35 and SF2/ASF have an antagonistic role in the splicing regulation of *MDM2*.

Our observations also support that changes in the levels and cellular localization of these SR proteins might well be associated with how constitutive and alternative splicing of *MDM2* is regulated under normal and damage conditions. Considering all this, we propose a working model for the splicing regulation of *MDM2* under normal and stress conditions that might as well recapitulate the events taking place in cancer cells. Upon normal or homeostatic conditions, SC35 binds at exon 11 to coordinate the inclusion of this exon in the final mRNA product (Fig. 14A). However, in response to stress induced by damage conditions or in cancer, SF2/ASF, which we have shown is overexpressed in these conditions, binds to exon 11 to inhibit the inclusion of this exon (Fig. 14B). This in turn results in the generation of the *MDM2-ALT1* alternative spliced transcript that has extensively been implicated in cancer.
### Primers used in the construction of MDM2 3-11-12 406 minigene and ESE mutants

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tr>
<td>X3 del 1 BH1 F</td>
<td>CCACCTCACAGATTCGCCGG</td>
</tr>
<tr>
<td>X3 del 1 Pst R</td>
<td>CAAAATAACTACAGCTCG</td>
</tr>
<tr>
<td>I 10 del 1 Pst F</td>
<td>CTGATTGAAAGAAATAGGGCG</td>
</tr>
<tr>
<td>X1I MDM2ER1 R</td>
<td>GAAGCTGATATTGCTCTTAATCTTGG</td>
</tr>
<tr>
<td>Int del 4 ER1 F</td>
<td>GCTAGCACTTCTGACTGAGCAG</td>
</tr>
<tr>
<td>SC35 at 57 Fwd</td>
<td>CCACCCTACAGATTCTATTCGGAAACAGGACC</td>
</tr>
<tr>
<td>SC35 at 57 Rev</td>
<td>GTCTGTGTGTCTCGGAGATCTTGAGGCTG</td>
</tr>
<tr>
<td>SC35 at 73 Fwd</td>
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<td>SC35 at 73 Rev</td>
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</tr>
<tr>
<td>SC35 at 165 Fwd</td>
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</tr>
<tr>
<td>SC35 at 165 Rev</td>
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</tr>
<tr>
<td>SC35 at 213 Fwd</td>
<td>AGAATCTCGAGATGCTATTCGGAAGAGCTTGAT</td>
</tr>
<tr>
<td>SC35 at 213 Rev</td>
<td>GTATACCTACAGCTAGGAAATTTAAGGATCGCT</td>
</tr>
<tr>
<td>SC35 at 382 Fwd</td>
<td>GTCTTTATCATTGAGGACTATTGAAATGCTAGTC</td>
</tr>
<tr>
<td>SC35 at 382 Rev</td>
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<tr>
<td>SC35 at 165 comp Fwd</td>
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<td>SC35 at 165 comp Rev</td>
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<td>SC35 at 213 comp Fwd</td>
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<tr>
<td>SC35 at 213 comp Rev</td>
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</tr>
<tr>
<td>SF2/ASF at 175 Fwd</td>
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<td>SF2/ASF at 175 comp Rev</td>
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<tr>
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<tr>
<td>SF2/ASF at 175 S1 #2 Rev</td>
<td>CTGATACCTACAGGAGGGGAGAGTGATACAG</td>
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Table 1. Outline of the primers used in the construction of MDM2 3-11-12 406 minigene and ESE mutants.
Figure 7. MDM2 minigenes undergo damage induced alternative splicing in vivo. A. Illustration of the 3-4-10-11-12 and 3-10-11-12 MDM2 minigenes. The exons are represented by boxes whereas the introns are depicted by lines and their corresponding sizes have been included in the diagram. The shaded region preceding exon 3 corresponds to the FLAG tag sequence. B) H1299, MCF7 and Hela cell lines were transfected with the indicated minigenes. Cells were exposed to 50J/m² of UVC after 20 hours of been transfected and total RNA was harvested 24 hours post treatment. Total RNA was submitted to RT-PCR and restricted detection of spliced transcripts from the MDM2 minigenes was achieved through amplification using primers specific for the FLAG tag and MDM2 exon 12. C. Same description as for B. The genotoxic agent used in this experiment is Cisplatin. The engineered MDM2 minigenes are capable to undergo damage induced alternative splicing in response to UVC (B) and Cisplatin (C) treatment. Therefore, these splicing competent minigenes contain the required elements that drive the damage induced regulation of MDM2's alternative splicing. (This figure was developed through the collaboration of Ravi K. Singh and Dawn S. Chandler).
Figure 8. The MDM2 3-11-12 406 minigene contains conserved ESE regulatory sequences for the binding of SR protein SC35 in exons 3, 11 and 12. A. Diagram of the 3-11-12 406 minigene. The exons are depicted by boxes whereas the introns are depicted by lines. The sizes for both exons and intron have been also outlined in this figure. The red box in exon 3 represents the FLAG tag sequence. The conserved SC35 exonic splicing enhancers (ESEs) in exon 3, 11 and 12 are illustrated by the green bars and they show their relative localization in the corresponding exon. B. The table outlines the conserved SC35 ESEs by its localization in the MDM2 3-11-12 406 minigene and we describe the specific nucleotide sequence followed by its predicted ESE value. Also, indicated in bold are the nucleotides mutated to disrupt the SC35 ESEs and the corresponding predicted ESE values after in vitro site directed mutagenesis. C. The table indicates the compensatory mutations engineered to re establish the ESE values for the SC35 ESEs 165 and 213. The compensated sequences show the new altered nucleotides in bold green and the predicted ESE values after the introduction of the compensatory mutation. (This figure was developed through the collaboration of Ravi K. Singh).
Figure 9. Exonic splicing enhancers in MDM2 3-11-12 406 regulate the inclusion of exon 11 through the function of SC35. A. MCF7 cells were individually transfected with MDM2 3-11-12 406 minigenes that contain mutated SC35 ESEs 57, 165, 213, 382 and 73. After 20 hours of transfection, the (+) cells were treated with 50J/m² and total RNA was harvested 24 hours post treatment. The RNA was submitted to RT-PCR and restricted detection of spliced transcripts from the MDM2 minigene was achieved using specific primers for the FLAG tag sequence and exon 12. The MDM2 3-11-12 406 minigene was used as a control and we show that this new minigene retains regulatory cis-acting elements that support the stress induced alternative splicing of MDM2. An increase in the skipping of exon 11 and consequently in the MDM2-ALT1 form was observed in cells untreated and treated with UVC and transfected with the 3-11-12 406 minigene mutants for SC35 ESE 165 and 213. The 3-11-12 406 minigene mutants for SC35 ESE 57, 382 and 73 didn’t showed a change in the splicing pattern in comparison to the control. B. Compensatory mutations were introduced to carry out a validation experiment that would further confirm the role of the conserved ESEs in MDM2 splicing. Both SC35 ESE comp 165 and 213 could partially rescue the splicing pattern disrupted by the introduced disrupting mutations at these ESEs. (This figure was developed through the collaboration of Ravi K. Singh).
Figure 10. The *MDM2* 3-11-12 406 minigene contains conserved ESE regulatory sequences for the binding of SR protein SF2/ASF in exons 3 and 11. A. Schematic of the *MDM2* 3-11-12 406 minigene with predicted SF2/ASF ESEs (orange bars). B. Description of ESE 175 with the two predicted binding sites for SF2/ASF, site #1 and site #2. Nucleotides common to both sites appear in black while in blue and orange we show dinucleotides that provide predicted differential binding either at site #1 or #2 correspondingly. C. Outline of the SF2/ASF ESEs sequence and their predicted ESE value before and after the mutations. The nucleotides altered to disrupt the ESEs are indicated in bold. D. The compensated sequence for the 175 ESE is described. The compensatory nucleotide alteration is shown in bold green. This compensatory mutation combination was the only possible one that could re-establish ESE values to the original while keeping the first disruption. Note that still, this compensatory mutation would only re-establish predicted ESE values for the binding of SF2/ASF at site #2.
Figure 11. An exonic splicing enhancer in MDM2 3-11-12 406 regulates the exclusion of exon 11 through the function of SF/2ASF. A. MCF 7 cells transfected with the MDM2 3-11-12 406 minigene mutated at ESE 48 and 175. After 20 hours of transfection, the (+) cells were treated with 50J/m² and total RNA was harvested 24 hours post treatment. The RNA was submitted to RT-PCR and restricted detection of spliced transcripts from the MDM2 minigene was achieved using specific primers for the FLAG tag sequence and exon 12. The MDM2 3-11-12 406 minigene was used as a control. We observed an increase in the inclusion of exon 11 in cells transfected with the mutated ESE 175 upon treatment and non treatment with UVC. The switch to exon 11 inclusion is drastic since the MDM2-ALT1 form is almost undetectable. The MDM2 3-11-12 406 minigene mutated at ESE 48 didn’t show any change in the splicing pattern compare to the control. B. Compensatory mutations were introduced to carry out a validation experiment that would further confirm the role of the conserved ESE 175 in MDM2 splicing. However, the compensatory mutation in ESE175 fails to rescue the splicing pattern and show no difference in compare to the mutated ESE 175.
Figure 12. The SF2/ASF ESE 175 Site #1 regulates exclusion of exon 11. A. The table displays the new mutations in ESE175 that would disrupt site #1, site #2 and site #1 and site #2. Experiments were carried out as previously described. B. The MDM2 3-11-12 406 minigene and the same minigene harboring the originally mutated ESE 175 were used as a control. Both controls recapitulated the previously observed results. We observed an increase in the inclusion of exon 11 in cells transfected with the mutated ESE 175 site #1 minigene upon treatment and non treatment with UVC. This splicing pattern resembles the splicing pattern observed with the mutated ESE 175. However, the mutated ESE 175 site #2 shows a splicing pattern that resembles the one from the unmutated 3-11-12 406 minigene control. Mutation of both site #1 and site #2 also showed inclusion of exon 11 due to the disruption of site 1.
Figure 13. The splicing pattern of MDM2 3-11-12 406 minigene cannot be rescued after disruption of SF2/ASF ESE 175 site #1. A. The table describes the second mutation introduced for disruption of ESE 175 site #1, the ESE value after the mutation and after the introduction of the compensatory mutation that is also outlined. B. Experiments were carried out in MCF7 cells as previously described. The second mutation to disrupt ESE 175 site #1 also results in the inclusion of exon 11 under treatment and not treatment conditions. However, a second attempt to compensate for the introduction of this mutation failed to re establish the splicing pattern observed for the unmutated minigene.
Figure 14. Working model for the role conserved ESEs and trans-acting factors in alternative splicing of MDM2. Based on observations from experiments carried out in our lab and in the findings reported in this manuscript we present a working model for the role of conserved ESEs and splicing factors SC35 and SF2/ASF in the regulation of splicing and alternative splicing of MDM2. A. Upon normal homeostatic conditions SC35 participates in the exon definition process binding exon 11 at the identified conserved ESE’s and identifying exon 11 as an exon to be included in the final mRNA product. B. However, upon conditions of stress-induced DNA damage and where we have observed that SC35 redistributes to larger and fewer nuclear speckles, the splicing factor SF2/ASF binds the identified conserved ESE 175 at exon 11 antagonizing the recognition of this exon by SC35. As a result exon 11 is spliced out in the final mRNA product and MDM2-ALT1 is generated.
Chapter 3: An *in vivo* model to determine the role of MDM2-ALT1 in the emergence of B cell malignancies.

3.1 Introduction

MDM2 can regulate cell fate under homeostatic and DNA damage conditions and upon oncogenic insult triggered by misregulation of oncogenes. Under homeostatic conditions MDM2 promotes cellular proliferation through its interaction with the tumor suppressor protein p53. However, this interaction is disrupted through the post transcriptional modification of both proteins in response to DNA damage which leads to p53 transcriptional activation and ultimately to cell cycle arrest and/or apoptosis (APPELLA and ANDERSON 2001; BROOKS and GU 2003). Interestingly, MDM2-ALT1, an MDM2 splice variant, is induced in response to DNA damage and its interaction with full length MDM2 correlates with the activation of p53 and its downstream targets (CHANDLER *et al.* 2006a; EVANS *et al.* 2001).

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2 The data presented in this chapter was partially generated by Lisa Caldwell, Reeva Aggarawal, Thomas W. Bebee, Jordan T. Gladman and Aixa S. Tapia-Santos. Acknowledgement for their specific collaboration in the generation of figures in this manuscript is stated throughout the figure section in this chapter.
Similarly, upon oncogenic insult, inhibition of MDM2’s negative regulation of p53 occurs through the binding of MDM2 to ARF (WEBER et al. 2000). Considering this, MDM2, through its interaction with MDM2-ALT1 and ARF can promote cell cycle arrest and or apoptosis. Consequently, integral regulation of MDM2 is critical to trigger the cellular protective response that is initiated upon DNA damage and oncogenic insult and to determine cell fate upon those cellular contexts. Indeed, it has been found that MDM2 is amplified and/or overexpressed in many human cancers where also many alternative spliced forms have been detected. Therefore, thorough investigation about the misregulation of MDM2 and the role of its alternative spliced forms should provide significant insight about the events that drive tumorigenesis in the tissues where these characteristics have been found.

Since their discovery in 1996, at least ten different MDM2 spliced variants have been identified in tumors and in normal tissues (BARTEL et al. 2004; JEYARAJ et al. 2009; VOLK et al. 2009). Moreover, alternative spliced forms of MDM2 have been detected in B cell malignancies like leukemias and lymphomas (BUESO-RAMOS et al. 1995; SANCHEZ-AGUILERA et al. 2006). However, the attempts to identify a role for these spliced variants have been controversial since the beginning. Upon their initial discovery, cloned alternative spliced forms of MDM2 that were detected in human tumor samples showed their ability to generate transformed foci at high frequency when transfected in to NIH3T3 cells (SIGALAS et al. 1996). On contrast to this finding, reports from Dang et al. showed that
several MDM2 splice variants could inhibit cell proliferation in a p53 dependent manner (Dang et al. 2002). However, specific research focusing in, Mdm2-b the murine version of MDM2-ALT1 has shown by in vitro experiments that this spliced form can promote cell growth and proliferation of NIH3T3 cells and of MEF’s that are null either for p53, ARF and Rb. The same report also showed that transgenic mice expressing this isoform under the control of a GFAP promoter results in the induction of tumors between 50-100 weeks (Steinman et al. 2004). Moreover, an additional independent study showed that tail vein injection of C57BL/6 recipient mice with Eµ-myc HSC’s transduced with a vector expressing an MDM2-ALT1 like protein (∆28-298) results in accelerated lymphomagenesis (Fridman et al. 2003). However, the extensive detection of MDM2-ALT1 in tumors and the effects of its expression in the above described in vivo experiments are inconsistent with its reported mechanism of action, where upon a complex formation with MDM2 full length, p53 activation is observed. So far many reports have made suggestions about how MDM2-ALT1 could possibly have a role in transformation while still keeping its ability of modulating p53. However, still no scientific evidence has risen to provide a compounding mechanism of action for the role of MDM2-ALT1 in tumors. All of the above suggests that at least some MDM2 alternative spliced variants, MDM2-ALT1 being one of them, contribute to the events leading to tumorigenesis and that assessing their role in a significant in vivo model system is imperative to elucidate their mechanism of action in disease.
The c-Myc oncogene is a transcription factor with critical roles in the normal cell cycle progression of cells. Interestingly, activation of Myc under like nutrient starvation can trigger an apoptotic program that can be dependent or independent on the p53 pathway (DANG 1999). Like MDM2, c-Myc is amplified and overexpressed in many human cancers including B-cell lymphomas lung carcinoma, breast carcinoma and some colon carcinomas (ARRATE et al. 2010; LITTLE et al. 1983; MARIANI-COSTANTINI et al. 1988; MUNZEL et al. 1991). A study aiming to identify effectors of c-Myc induced pathways promoting transformation or apoptosis showed that ARF and p53 mediate c-Myc induced apoptosis in primary pre-B cells. The authors demonstrated that p53/-, ARF/- and p53/-ARF/- pre-B cells from over expressing c-Myc transgenic mice are resistant to apoptosis. The same authors showed that tumors from mice overexpressing c-Myc undergo inactivation of p53 either by mutations in p53 or by loss of ARF. With this it was proposed that the overexpression of c-Myc selects for the inactivation of the ARF-DM2-p53 pathway inhibiting the protective apoptotic function of c-Myc and promoting transformation (EISCHEN et al. 1999). Based on our own observations of the function of MDM2-ALT1 and in the extensive reports that still associate this MDM2 spliced form with tumorigenesis, we propose a similar mechanism for the tumorigenic potential of MDM2-ALT1. In this model, the overexpression of MDM2-ALT1 triggers a protective program in which by an interaction with full length MDM2, p53 becomes transcriptionally active to drive the expression of downstream target genes involved in cell cycle arrest and/or
apoptosis. However, persistent expression of MDM2-ALT1 results in the abrogation of the protective program due to MDM2-ALT1 induced acquisition of spontaneous mutations in the p53 pathway that allow for the proliferation of misregulated cells (Fig. 15).

The current information from both in vitro and in vivo experiments on MDM2-ALT1 seems inconclusive due to the lack of a clear mechanism of action that explains its dual role in cellular protection and tumorigenesis. Because MDM2-ALT1 is the most common alternative spliced form detected in human and murine tumors and because identification of its role in tumorigenesis could prove to be relevant in the development of therapeutics targeting both alternative splicing and the MDM2-p53 pathway, we have developed a transgenic mouse model for the study of MDM2-ALT1 in vivo. Even more, knowing from other in vivo experimental approaches, that the expression of MDM2-ALT1 is lethal but moreover that cellular context in which the expression of MDM2 spliced forms takes place appears to be determining for their mechanism of action, we have decided to generate an inducible mouse model that would restrict the expression of MDM2-ALT1 to B cells. Because the endogenous expression of MDM2-ALT1 in tumors of B cells has been well established, our mouse model will faithfully recapitulate the in vivo events driving cells to undergo transformation upon the expression of this spliced form. Most importantly, eventually, this mouse model will be useful to carry out an in vivo analysis of the effects of MDM2-ALT1
expression in different cellular contexts and in cells that have different genetic backgrounds relevant for the role of MDM2-ALT1 (i.e. p53-/-, ARF-/-, MDM2+/+).

In this study we show that we have successfully developed a transgenic mouse model in which MDM2-ALT1 expression is restricted to B cells. We have analyzed our mice for survival and disease onset. We report that there are no significant differences in survival rates or tumor spectrum between the MDM2-ALT1 transgenic positive mice and the control mice. However, leukemia was only detected in two MDM2-ALT1 transgenic mice and the emergence of degenerative joint disease was significant in the transgene positive group. We will utilize our MDM2-ALT1 mouse to carry out a more comprehensive study in which we will additionally analyze the result of expressing this isoform in mice that have different genetic backgrounds (p53, ARF null).

3.2 Materials and Methods

**Generation of the transgenic MDM2-ALT1/CD19 Cre mice.** The *MDM2-ALT1* transgene was generated by cloning of the human cDNA sequence in to the mammalian expression vector pCCALL2 (LOBE et al. 1999). Transcription of the transgene in this vector is driven by the cytomegalovirus (CMV) promoter and the chicken β-actin enhancer and is dependent on *Cre* recombinase excision of a βgeo cassette that is upstream of the *MDM2-ALT1* sequence. The functionality of the promoter in our transgenic construct was verified by transfection of NIH3T3
murine fibroblast cells (American Type Culture Collection, Manassas, VA) and β-gal staining (NAGY et al. 2003). To confirm that our transgenic construct was capable of undergoing Cre-mediated recombination, E. coli BNN132, a bacterial strain that expresses the Cre-recombinase enzyme, was transformed with the pCALL2/MDM2-ALT1 transgene and plasmid DNA from individual colonies was digested with Pst1 restriction enzyme to visualize fragments indicating recombination on an agarose gel. The pCALL2/MDM2-ALT1 construct was linearized with Stu1 restriction enzyme and purified by standard methods (NAGY et al. 2003). The transgenic mice were generated by electroporation of the linearized pCALL2/MDM2-ALT1 construct into ES cells. This procedure was carried out by the Genetically Engineered Mouse Facility at The Research Institute at Nationwide Children’s Hospital. Electroporated ES cells were maintained in G418 containing medium for selection of transgene expressing cells. ES cell resistant colonies were screened by β-gal staining and Southern analysis of Eco RV digested genomic DNA following established methods (NAGY et al. 2003). Cell lines with a single integration event where selected for blastocyst injection (2C12 and 1C8). Chimeric mice were crossed to C57BL/6 mice to generate F1 heterozygotes. Germline transmission of the transgene was determined by PCR. PCR primers used for genotyping span the X3-X12 junction to the PolyA region, HDM2 X3 Z12junS forward 5’-GAGACCCTGGACTATTGG-3’ and PolyA reverse 5’-CCCCATAATTTTTGAGGAG-3’. F1 heterozygotes were backcrossed to C57BL/6 to generate F3 heterozygotes that where crossed
to CD19Cre- mice to generate the double transgenic MDM2-ALT1 / CD19Cre mice used in this study. The resulting double transgenic mice were genotyped for expression of the *MDM2-ALT1* transgene using the above described set of primers and for expression CD19 Cre- recombinase using the PCR primers SOX2Cre- Fwd 5’-CCTGTTTTGCACGTTTACCC-3’ and SOX2Cre- Rev 5’-ATGCTTCTGTTTCCGTTTACCC-3’. RNA extraction from spleen and muscle was carried out using an RNeasy mini kit (Qiagen, Valencia, CA). cDNA was generated using Transcriptor reverse transcriptase (Roche Diagnostics, Indianapolis, IN) with random hexamer primers. To evaluate transcription of the MDM2-ALT1 transgene in B cells from the transgenic mice spleens, the cDNA was submitted to PCR using the HDM2 X3 Z12junS and PolyA reverse primer set previously described in this section. PCR products were visualized on an electrophoresed 2% agarose gel.

**Pathological analysis of transgenic mice.** Mice were monitored for tumor development by weekly and biweekly palpation of lymph nodes. The duration of the screening was 2 years and 21 days. Mice showing signs of pain or distress or in which masses, abscesses and tumors were evident were humanely euthanized in a CO2 chamber following IACUC guidelines. Euthanized mice were necropsied and examined for lesions and tissue abnormalities. Tissue specimens (spleen, lung, liver, kidney and any other tissue of interest including tumors) were fixed in 10% neutral buffered formalin. Paraffin embedding of tissues, sectioning, H&E staining, B220 and CyclinD1 immunohistochemistry and
pathological analysis were carried out by the Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University Department of Veterinary Medicine. Kaplan-Meier statistic analysis and graphic representation of results obtained were carried out using GraphPad Prism (version 5.0 for MacOX, GraphPad Software, San Diego, CA).

**Harvesting of splenocytes for flow cytometry analysis.** Spleens were removed from euthanized mice and splenocytes were harvested by pounding of the spleen using a 12-well plate smashing device generously provided by Dr. Santiago Partida-Sanchez. The procedure was carried out in ice using 1X PBS or FACS buffer as the suspension solution. Red blood cells were lysed by treatment and incubation of the splenocytes in ACK lysis buffer (Invitrogen, CA). Cells were washed in 1XPBS or FACS buffer and used for flow cytometry analysis. Trypan blue cell counting was carried out and 2X10^6 splenic cells from individual mice were plated for multicolor immunofluorescence staining. Non specific binding of antibodies was blocked by pre-incubation of cells with anti-mouse CD16/CD32 antibody (e Bioscience, Inc). We limit our flow cytometry analysis to live cells using LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit (Invitrogen, CA). The anti-mouse fluorescent conjugated antibodies used in this analysis are: PE-anti CD-19, FITC-anti IgM (eBioscience, Inc), PerCP anti-CD5, PE-Cy^TM^5 anti-TCR β chain and V450 anti-CD45 (BD Biosciences). Staining of cells was carried out by incubation (15 minutes) in a pre mixed solution in which the eBioscience conjugated antibodies were used in 1:200 dilution and the BD Biosciences were
used in a 1:100 dilution. After incubation cells were centrifuged for five minutes at 1,200 RPM’s and rinsed with FACS buffer. Stained cells were analyzed no later than 30 minutes post staining at The Research Institute Flow Cytometry Core Laboratory using a BD-LSRII flow cytometer (BD Bioscience). Generated data was further analyzed using FlowJo flow cytometry analysis software (TreeStar, Inc., OR).

3.3 Results

Generation of a tissue specific transgenic MDM2-ALT1 mouse model. It has been previously shown in pronuclear injection experiments that ubiquitous expression of Mdm2-b, the murine homologue of MDM2-ALT1, results in embryonic lethality (STEINMAN et al. 2004). This is consistent with the role of MDM2-ALT1 activation of the p53 apoptotic pathway. Therefore, to overcome this difficulty we have opted to generate a tissue specific MDM2-ALT1 transgenic mouse in which the expression is restricted to B cells. To achieve this we have modified the well established Z/AP double reporter construct to drive the expression of MDM2-ALT1 upon expression of the Cre-recombinase enzyme (LOBE et al. 1999). The pCALL2 vector contains the pCAGG vector promoter which consists of the CMV enhancer and the chicken β-actin promoter followed by the β-geo cassette and three copies of the SV40 poly adenylation sequence (3XpA) (NIWA et al. 1991). The β-geo cassette expresses both the neomycin
resistance gene (neo) and the lacZ reporter gene to allow for selection of cells harboring the construct and for determination of recombination efficiency correspondingly. Both the β-geo cassette and the 3XpA are flanked by loxP sites and they are excised upon Cre- recombinase mediated recombination (Fig. 16A). This system has been well characterized in mice and shows efficient Cre-recombinase mediated removal of the beta geo cassette and subsequent expression of the downstream gene of interest without leaky expression (LOBE et al. 1999). We have created the MDM2-ALT1 transgene by cloning the human MDM2 cDNA sequence corresponding to the MDM2-ALT1 spliced form into the pCALL2 vector (Fig. 16A). To assess the ability of the newly generated pCALL2/MDM2-ALT1 construct to express lacZ, we transfected murine NIH3T3 cells with this construct and with the unmodified pCALL2 vector as a control. β-gal staining of the transfected cells shows positive staining for lacZ activity in cells transfected with both the unmodified pCALL2 vector and the pCALL2/MDM2-ALT1 construct but not in cells mock transfected with no DNA (Fig. 16B). To confirm that the loxP sites in the pCALL2/MDM2-ALT1 construct can efficiently mediate Cre- recombination we transformed E. coli BNN 132 cells with the pCALL2/MDM2-ALT1 plasmid. pCALL2/MDM2-ALT1 plasmid DNA was isolated from single bacterial colonies and digested with the restriction enzyme PstI. Digested DNA was then run on an agarose gel to visualize the resultant fragments. We have outlined a strategy for detection of efficient recombination in transformed E. coli cells after PstI digestion (Fig. 17A-B). We show that in four of
the six transformants excision of the β-geo cassette took place and therefore the integrity and competency of the Cre- recognition sequences is maintained in the pCALL2/MDM2-ALT1 construct (Fig. 17C). Moreover, expression of MDM2-ALT1 upon Cre-recombinase mediated recombination was shown by western blot analysis of protein lysates obtained from NIH3T3 cells transfected with recombined pCALL2/MDM2-ALT1 plasmids (Fig. 17D). Therefore these experiments show that the pCALL2/MDM2-ALT1 construct can effectively drive the expression of both the β-geo cassette and MDM2-ALT1 upon Cre- mediated recombination.

Generation of transgenic mice by Embryonic Stem (ES) cell-mediated transgenesis provides an alternative for producing transgenic mice when there is a need for low copy number (single copy) integration (Nagy et al. 2003). We have turned to this approach for the generation of our transgenic MDM2-ALT1 mouse model. ES cells electroporated with the pCALL2/MDM2-ALT1 construct were maintained in G418 containing medium to identify correctly transgene containing ES cell lines. 288 G418 resistant ES cell colonies were propagated for initial screening. ES cells were screened by Southern blot analysis and by β-gal staining (Fig. 18A-B). From the analyzed cell lines, seven showed to have a single integration event and from those, four were chosen for further characterization (1C8, 2F12, 3B12, and 2C12). The ability of Cre- to effectively recombine loxP sites can be affected by the integration sites of individual transgenes. To confirm that these cell lines remained competent for Cre-
mediated recombination after the integration event, we electroporated these ES cell lines with a Cre-recombinase expression plasmid and scored them for recombination. We show that the four cell lines retained their ability to undergo Cre-recombination (Fig. 18C). The 2C12 and 1C8 ES cell lines were selected for blastocyst injection and generation of chimeric mice. Resultant chimeric mice were crossed to C57BL/6 mice to produce F1 heterozygotes. We confirmed by PCR that the 2C12 and 1C8 cell lines have effectively transmitted the MDM2-ALT1 transgene to the germline (Fig. 19A). Two additional backcrosses to C57BL/6 mice were carried out to generate the F3 heterozygote mouse colonies. We have then crossed our F3 2C12-MDM2-ALT1 and F3 1C8-MDM2-ALT1 to mice expressing Cre-recombinase under the control of the CD19 promoter. The resultant MDM2-ALT1/CD19Cre- mice will express the MDM2-ALT1 transgene exclusively in B-cells and they have been genotyped for both the transgene and Cre-recombinase (Fig. 19B-C). To verify the expression of the MDM2-ALT1 transcript in our mice we isolated total RNA from the spleen and muscle of 2C12 transgene positive (Tg+) and CD19Cre positive mice (CD19+), as well as transgene negative (Tg-) and CD19Cre positive (CD19+) mice. RT-PCR was carried out using primers specific for the detection of MDM2-ALT1 transcripts. As expected, our experiment shows that transcription of the 2C12 MDM2-ALT1 transgene is taking place in the Tg+ CD19+ mice but not in the Tg- CD+ mice and that the expression of the transgene is restricted to tissue expressing Cre-recombinase (spleen= high number of B cells) (Fig. 20). We have also
sequenced the MDM2-ALT1 transgene in our 2C12 mice using gDNA isolated from their tails to verify that the transgene integrity has not being altered. Sequencing results showed that the transgene sequence has remained unaltered since no deletions or nucleotide substitutions were detected.

The above described experiments and results show that our transgenic 2C12 MDM2-ALT1+/CD19Cre- mice undergo transcription of the transgene in tissue where a high number of B cells is expected. Therefore, we have successfully developed an in vivo model in which the effects of MDM2-ALT1 in B cells can be assessed.

**Pathological analysis of MDM2-ALT1/CD19Cre mice.** To assess the role of MDM2-ALT1 in vivo, the 2C12 MDM2-ALT1/CD19Cre mice were monitored for solid tumor development for a maximum period of two years and twenty one days. The screening was carried out weekly for approximately one year and four months and biweekly for the last eight months of the study. Every mice was screened by palpation of lymph nodes, chest and abdominal area. Mice bearing suspicious masses, moribund or showing signs of distress were euthanized in a CO2 chamber to comply with established IACUC guidelines. We recorded the body weight and the spleen weight of the mice prior to necropsy to identify differences that could be indicative of solid tumor load and to establish a spleen:body weight ratio that could be preliminary used as an indicative of proliferation differences in the spleen (Tables 2-3). We found that there are no significant differences between the body weights of transgenic positive mice and
transgenic negative mice from the same sex (Fig. 21A-B). However, male control mice that did not express the MDM2-ALT1 transgene show significant increased spleen weight whereas the spleen weight in males that are transgene positive appears to be reduced when compared to male mice expressing the transgene (Fig. 21C). In line with this, we observed a significant increase in the spleen:body weight ratio in male control mice compared to male mice expressing MDM2-ALT1 (Fig. 21E). However, no significant differences in spleen weight or in the spleen:body weight ratio were apparent between control and transgene positive females, though these results are confounded by the low number of female mice in this study (Fig. 21D, F). The effect of MDM2-ALT1 in survival was also analyzed (Table 2-3). We show that there were no significant differences in survival between the MDM2-ALT1+ mice and the MDM2-ALT1- mice since a difference of approximately 37 days was observed (Fig. 21G). Mice were necropsied either at our facility or at the Comparative Pathology and Mouse Phenotyping Shared Resource (CPMPSR) at The Ohio State University Department of Veterinary Medicine. Tissues (spleen, liver, kidney and any suspicious tissue) were fixed in 10% formalin and submitted to the CPMPSR. H&E staining and immunohistochemistry (IHC) of fixed tissues was also carried out by this facility. The CD45R B220 marker is useful in the identification of proliferating B cells and CyclinD1 expression is associated with cell cycle G1/S transition through the regulation of CDK4 and CDK6. Moreover, antibodies targeting these proteins are commonly utilized in IHC staining to diagnose mantle
cell lymphoma, B-cell chronic leukemia (B-CLL) and hairy cell leukemia among other B cell malignancies. For IHC, antibodies anti CD45R B220 and anti CyclinD1 were used. Therefore, to identify differences in cell cycle and proliferation and possible B cell malignancies antibodies targeting these proteins were used in IHC. Dr. Krista La Perle from the CPMPSR, carried out the pathological analysis of the mice under this study and table 4 outlines the distribution of the analyzed mice by group.

We have carried out statistical analysis from the pathological results using GraphPad Prism software. The pathological analysis resulted in the detection of lymphomas in both the control MDM2-ALT1-/CD19Cre+ and the MDM2-ALT1+/CD19Cre+ groups. No significant differences between the groups were found for the emergence of lymphomas. Eventhough leukemia and hemangiosarcoma were detected only in mice expressing MDM2-ALT1, no significant results arise from this finding when establishing a comparison with transgene negative control mice. Similarly, skin carcinomas and other malignancies, including an hepatocellular carcinoma and a pituitary gland adenoma where comparable in both groups. Interestingly, the pathological analysis indicated that two of the MDM2-ALT1+ mice showed germinal center formation in the spleen whereas no germinal center formation was reported for any of the control mice. In conclusion, no statistical relevant differences in tumorigenesis emerged from the pathological analysis of the mice in this study. However, because our mouse cohort was relatively small and not in all of the
mice we were able to carry out a complete pathological analysis, a second study in which the mouse number is increased and comprehensively analyzed by pathological methods could yield better results.

**Flow cytometry analysis of splenic B cells.** B cells can be characterized by conducting a flow cytometric immunophenotyping assay in which the expression of proteins can be assessed. Recent reports have shown that B cell chronic lymphocytic leukemia (B-CLL), the most common hematologic malignancy in adults in the Western world, is characterized by clonal expansion of B cells expressing the Cluster of Differentiation 5 (CD5) protein (CAIRO et al. 2010; EFANOV et al. 2010; PEKARKSY et al. 2010; SANTANAM et al. 2010). These reports describe a B-CLL malignant phenotype as CD19+, IgM+ and CD5+. We used flow cytometry to determine the immunophenotypic profile of splenic B cells from MDM2-ALT1+/CD19Cre+ mice. Multicolor flow cytometry was carried out using fluorescence conjugated antibodies targeting the T-cell receptor, CD19, IgM and CD5 and the flow cytometry raw data was analyzed using FlowJo software. In both our experimental method and in our analysis, we discriminated between dead and live cells to rule out the possibility of mistakenly accounting false positive events that can result from non specific interactions. To distinguish B cells we analyzed the splenocytes for the expression of the T-cell receptor and CD19. The cell population positive for the expression of CD19 was gated and further analyzed for the expression of IgM and CD5. Eventhough, we carried out flow cytometry on 12 of the mice, (3 controls and 9 transgenic positive), we only
obtained conclusive results for 4 transgenic positive and 1 BL6 age control mouse. This was in some cases due to experiment difficulties (i.e. mice Agouti, 11 and 30, didn’t show staining of B cells) or due to abnormal findings in mice (i.e. mouse #31 spleen was hardened to the point that it could not be smashed, lymphoma was reported on this mouse in the pathological report). We report that from the 4 transgenic positive mice in which conclusive results were obtained, 1 mouse showed a subpopulation of B cells that appear to be positive for the expression of the CD5 marker (Fig. 23B). Interestingly, this mouse was one of two mice that was reported to have lymphoid hyperplasia with splenic germinal center formation. Although, additional experiments should be carried out in order to establish a formal diagnosis, this finding suggests that in these mice a B-CLL diagnosis might be possible.

3.4 Discussion

We have developed a mouse model to study the expression of MDM2-ALT1 in B cells. As part of this study we have proposed, a mechanism for the tumorigenic pathway in cells that persistently express MDM2-ALT1 (Fig. 15). In this pathway, the overexpression of MDM2-ALT1 promotes the acquisition of mutations in the p53 pathway and confers a growth advantage to cells harboring such mutations. Mutated cells with that growth advantage are able to proliferate and induced transformation due to the MDM2-ALT1 induced inactivation of p53.
We report that MDM2-ALT1+ males have reduced spleen weight and spleen:body weight ratio compared to transgene negative control male mice which have increased spleen weight. It might be possible that this observation is related to a protective action of MDM2-ALT1 in which splenic B cell proliferation is inhibited in comparison to mice that do not express the transgene. To either confirm or disregard this idea, it would be relevant to carry out experiments to determine the proliferation rate in B cells from MDM2-ALT1 expressing mice. No differences in survival were also observed between the groups and this is consistent with the lack of significant differences in tumorigenesis between the groups. Although, the emergence of leukemia and hemangiosarcoma was restricted to the MDM2-ALT1+ group, the number of events were not enough to establish significant statistical results. However, the finding that degenerative joint disease is prevalent and significant in MDM2-ALT1+ mice, although conflicting with the previous idea that MDM2-ALT1 expression is inhibiting proliferation in splenic B cells, is interesting due to the association of this disease with the clonal expansion of B cells. Degenerative joint disease or osteoarthritis is a non infectious chronic inflammatory disorder that affects the synovial joints. Although, the etiology and pathogenesis of this disorder is poorly understood recent studies have shown that the clonal expansion of B cells might be related with the inflammation component of the disorder (DA et al. 2007; ROLLÍN et al. 2008; SHIOKAWA et al. 2001). Also, one report has directly linked this described clonal expansion of B cells to mutations in the IgVH gene that affect the
complementarity-determining regions (CDR) (DA et al. 2007). Even more interesting is the fact that the mutational status of IgV$_H$ genes along with the expression of CD38 are prognostic markers in early stages of B-CLL where patients with hypermutated IgV$_H$ status have better prognosis than patients with unmutated IgV$_H$ (DAL-BO et al. 2011). Most importantly, a report in which the relationship between p53 dysfunction, CD38 expression and IgV$_H$ in B-CLL is studied, proposes several theories for the pathogenesis of B-CLL. Those theories go from the idea that dysfunction of the p53 pathway impairs the capacity of CLL cells to undergo hypermutation (poor prognosis) to the idea that the phenotype of tumor cells with extensively mutated IgV$_H$ genes might be altered in the presence of a p53 dysfunction, such that the disease is not recognizable as CLL (LIN et al. 2002). In any case, p53 dysfunction is key in both ideas and further investigation should be carried out to possibly associate MDM2-ALT1 expression to the p53 dysfunction that renders cells incapable to undergo hypermutation of the IgV$_H$ gene.
Figure 15. Model for the tumorigenic potential of MDM2-ALT1. We propose that similar to the published mechanism of Eµ-myc induced tumorigenesis, MDM2-ALT1, by its interaction with MDM2, initially mediates a protective period in which p53 is activated. However, upon persistent expression, MDM2-ALT1, induces the acquisition of mutations in the p53 pathway that confers cells with a growth advantage and the potential to undergo transformation.
Figure 16. The pCALL2/MDM2-ALT1 construct is capable of lacZ expression in NIH3T3 cells. A. Diagram of the pCALL2/MDM2-ALT1 construct. The diagram shows the pCAGG promoter, consisting of the CMV enhancer and the chicken β-actin promoter. It also shows the loxP flanked βgeo cassette and the 3X poly adenylation signal followed by MDM2-ALT1 sequence. Upon Cre-mediated recombination, the βgeo cassette and the 3X poly adenylation signal get excised and expression of the transgene takes place. B. The pCALL2 and the pCALL2/MDM2-ALT1 construct were transfected in NIH3T3 cells that were submitted to β-gal staining. We also carried out a mock transfection with no DNA as a control. We show positive staining for lacZ activity in both the pCALL2 vector and our pCALL2/MDM2-ALT1 construct. This experiment shows that our engineered pCALL2/MDM2-ALT1 construct can drive the expression of the βgeo cassette. (This figure was developed through the collaboration of Lisa Caldwell).
Figure 17. The pCALL2/MDM2-ALT1 construct is recombination competent and expresses the MDM2-ALT1 transgene. We outline a strategy for the detection of recombination in *E. coli* BNN132 cells transformed with the pCALL2/MDM2-ALT1 construct. The restriction enzyme *Pst I* digests the construct at two sites within the βgeo cassette and once outside of it. A. Upon lack of recombination and digestion of the construct with *Pst I*, three fragments of distinct sizes (7.5kb, 118bp and 2.8kb) should be detected upon separation of the fragments in an electrophoresed agarose gel. B. However, if recombination takes place only a 5.6kb fragment should be observed. C. The Cre-recombinase expressing bacterial strain *E. coli* BNN132 was transformed with the pCALL2-MDM2-ALT1 construct. Plasmid DNA was isolated from six single colony transformants and digested with *Pst I* restriction enzyme. Fragments were separated in an agarose gel and the results show that four of the six transformants underwent Cre-mediated excision of the βgeo cassette. D. NIH3T3 murine fibroblast cells where transfected with the pCALL2/MDM2-ALT1 (un recombed – no Cre) or with pCALL2/MDM2-ALT1 plasmids that have undergone recombination in *E. coli* BNN132 (+Cre). Protein lysates were prepared from these cells and analyzed by western blot using the HDM2 AF1244 anti MDM2 antibody. This antibody recognizes both full length MDM2 as well as the spliced form ALT-1. We show that recombined plasmids can effectively express MDM2-ALT1. (This figure was developed through the collaboration of Lisa Caldwell and Aishwarya Jacob.)
Figure 18. Screening of electroporated ES cells by βgal staining and southern blot analysis. We screened our electroporated ES cells for detection of positive cell lines that have effectively undergone integration of the MDM2-ALT1 transgene. A. ES cells were submitted to βgal staining and we show that electroporated ES cells show lacZ activity that is evident by the visual positive βgal staining. B. Southern blot analysis of several electroporated ES cells using a radiolabeled probed against the 3XpA sequence was carried out for detection of cell lines that have a single integration event of the transgene. We show that seven cell lines (*) are positive for the transgene integration event at a single site. (This figured was developed through the collaboration of Thomas W. Bebee).
Figure 19. Germline transmission of the MDM2-ALT1 transgene by the 1C8 and 2C12 cell lines and generation of the MDM2-ALT1/CD19Cre mice. A. Agouti pups from chimeric founder mice #2910-2C12 and #2909-1C8 were screened for the detection of the transgene by PCR using primers specific for the MDM2-ALT1 exon 3-12 junction and the Poly A sequence. C57BL/6 and chimeric founder mouse genomic DNA was used to generate negative and positive controls correspondingly. Mice positive for the transmission of the transgene produce a 629bp amplicon. F3 heterozygotes were generated for both the MDM2-ALT1 2C12 and 1C8 mouse lines by backcrossing the F1 heterozygotes to C57BL/6 mice. This F3 heterozygotes where crossed to CD19Cre mice to generate the MDM2-ALT1/CD19Cre mouse lines. B-C We screened the 2C12 and 1C8 MDM2-ALT1/CD19Cre mouse lines for harboring of the transgene as previously described. Additionally, we screened these mice for CD19Cre using specific primers described in the materials and methods section. We showed that we have successfully generated the MDM2-ALT1/CD19Cre mice for both mouse lines. (This figure was developed through the collaboration of Reeva Aggarawal).
Figure 20. MDM2/ALT1 is transcribed in CD19Cre active tissue from 2C12 MDM2-ALT1+/CD19Cre+ mice. 2C12 MDM2-ALT1+/CD19Cre+ (Tg+ CD19+) and MDM2-ALT1- /CD19Cre+ (Tg- CD19+) mice were sacrificed and total RNA was isolated from spleen (high number of B cells - Cre active) and from muscle (Cre inactive). RT-PCR was carried out and cDNA was amplified using specific primers for detection of MDM2-ALT1. We show that as expected, transcription of MDM2-ALT1 is taking place in the Tg+ CD19+ but not in the Tg- Cd19+ mouse and this is restricted to tissue that is active for the expression of Cre recombinase. (This figure was generated through the collaboration of Jordan T. Gladman).
### MDM2-ALT1 - / CD19Cre + mice included in the study

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**Table 2. Outline of the MDM2-ALT1 -/CD19Cre+ mice included in the study.** The table includes the recorded data used to generate the graphic representations and statistical analysis regarding the body weights, spleen weights and spleen:body weight ratio for the control mice group.
<table>
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<tr>
<th>Mouse Id</th>
<th>Sex (M/F)</th>
<th>Body Weight (g)</th>
<th>Spleen Weight (g)</th>
<th>Spleen / Body Weight (%BW)</th>
<th>Survival Time (Days)</th>
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</table>

Table 3. Outline of the MDM2-ALT1+/CD19Cre+ included in the study. The table includes the recorded data used to generate the graphic representations and statistical analysis regarding the body weights, spleen weights and spleen:body weight ratio for the experimental mice group.
Figure 21. Lack of MDM2-ALT1 affects spleen weight in males but does not affect survival. The body weight and the spleen weight was recorded for the 2C12 MDM2-ALT1-/CD19Cre+ and MDM2-ALT1+/CD19Cre+ mice. We also established the spleen:body weight ratio. A-B. No significant differences on body weight were observed between transgene positive and transgene negative mice of the same gender. C-E. Transgene negative male mice show a significant increase in the spleen body weight compare to transgene positive mice of the same gender. This increase in spleen weight in the transgene negative male mice affects their spleen:body weight ratio, where also a significant increase is observed in contrast to the transgene positive group. D, F. As opposed to male mice, no significant differences in spleen weight or in spleen:body weight ratio were observed between MDM2-ALT1 transgene positive and transgene negative female mice. G. There were no significant differences in the survival between the MDM2-ALT1+/CD19Cre+ and MDM2-ALT1-/CD19Cre+. 
Distribution of pathology and flow cytometry analysis of mice by group

<table>
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<tr>
<th>Genotype</th>
<th>Group</th>
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<th>Flow Cytometry</th>
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<td></td>
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<td>completed</td>
<td>pending</td>
<td>total</td>
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<tr>
<td>ALT1-/-CD19Cre+</td>
<td>Control</td>
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<td>3</td>
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<td>2</td>
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<tr>
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<td>Experimental</td>
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Table 4. Distribution of the pathology and flow cytometry analysis of mice by group. The table show the number of mice in which pathology analysis and/or flow cytometry was conducted.

Abnormalities found by pathology and flow cytometry analysis of mice by group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group</th>
<th>n</th>
<th>Lymphoma</th>
<th>Leukemia</th>
<th>Hemangiosarcoma</th>
<th>Skin Carcinoma</th>
<th>Other</th>
<th>n</th>
<th>Degenerative Joint Disease</th>
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<tbody>
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<td>Control</td>
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<td>3</td>
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<td>0</td>
<td>1</td>
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<td>7</td>
<td>1</td>
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<td>Experimental</td>
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<td>2</td>
<td>2*</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5. Abnormalities found by pathology and/or flow cytometry analysis of mice by group. The table outlines the abnormal findings after analysis of the mouse colony by pathological tests and/or flow cytometry. (* = 1 possible B-CLL).
Figure 22. Tumor spectrum in 2C12 MDM2-ALT1+/CD19Cre+ and control mice. A. Graphic representation of the malignancies reported in the pathological analysis. No significant differences in tumorigenesis were found between the groups. B. We report that degenerative joint disease is significantly prevalent in mice expressing MDM2-ALT1.
Figure 23. Identification of a CD5+ B cell sub population by flow cytometry analysis. Multicolor flow cytometric phenotyping was carried out to characterize splenic B cells from the MDM2-ALT1+ mice. Fluorescence antibodies targeting T cells (TCR), B cells (CD19), IgM and CD5 were used. A. Flow cytometry analysis of splenic B cells from an age control BL/6 mice show that these cells are CD19+, IgM+, CD5-. B. Flow cytometry analysis of splenic B cells from an MDM2-ALT1+/CD19Cre+ mouse shows a subpopulation of B cells that is positive for the expression of the CD5+ marker. Therefore these B cells show the reported CD19+, IgM+, CD5+ malignant immunophenotype.
Chapter 4. Summary and Discussion

Constitutive splicing is necessary for the generation of mature mRNA molecules that upon translation will produce integral and functional protein products. Both constitutive and alternative splicing are critical regulators of eukaryotic gene expression and the last is necessary for the maintenance of proteome diversity. These processes are govern by the interaction between regulatory cis-acting elements in the pre-mRNA molecule and the trans-acting factors that bind them. Therefore, sequence alterations affecting regulatory cis-acting elements and misregulation of trans-acting factors can adversely affect constitutive and alternative splicing and result in disease causing proteins. In that line, changes in the pattern of alternative splicing of transcripts have been associated with cancer (NOVOYATEVA et al. 2006). Interestingly, many cancer related genes, undergo changes in their splicing pattern without obvious mutations that can affect splice selection (GAVIN et al. 2003; KWABI-ADDO et al. 2001; PFEFFER et al. 1993; SHEN et al. 1991). Such events can be the result of misregulation of trans-acting splicing factors that have altered concentrations,
changes in their subcellular localization or phosphorylation state (RAFALSKA et al. 2004).

MDM2 is one cancer related gene that has been reported to undergo alternative splicing of its transcript without any apparent mutation affecting splice site selection (LUKAS et al. 2001; STEINMAN et al. 2004). The MDM2 protein is the main negative regulator of the tumor suppressor protein p53 and in response to stress and in cancer, alternative splicing of MDM2, generates an isoform, namely MDM2-ALT1 that can inhibit the negative regulation of MDM2 over p53. This results in the initiation of a cellular protective program in which transcriptional activation of p53 results on cell cycle arrest, DNA repair and/or apoptosis. However, although MDM2-ALT1, through its interaction with full length MDM2, can induce p53 dependent cell cycle arrest and apoptosis, its detection in many types of human cancers seems inconsistent.

In this study we have engaged into investigating the regulatory mechanism behind the alternative splicing of MDM2. We have shown that regulatory sequences in the form of exonic splicing enhancers (ESE’s) located at exon 11 of an MDM2 minigene can regulate the alternative splicing of MDM2 under normal and DNA damage conditions. We have identified two ESE’s for the predicted binding of the splicing factor SC35 and one ESE for the predicted binding of the splicing factor SF2/ASF. Interestingly, the three ESE’s are located at exon 11 of the MDM2 minigene, which suggest that the differential inclusion or exclusion of this exon is highly regulated. Evenmore, the resulting splicing
pattern after the introduction of mutations in this ESE’s suggests that SC35 and SF2/ASF through the interaction with these ESE’s have opposing roles in the regulation of MDM2 alternative splicing. We observed that when SC35 ESE’s are disrupted the splicing pattern favored excludes exon 11 from the final spliced product. This suggests that SC35 can positively regulate splicing of MDM2 through the inclusion of exon 11. The opposite was observed when the SF2/ASF ESE was disrupted, since the splicing pattern favored includes exon 11 in the final spliced product. In this way, the predicted SF2/ASF binding site is responsible for the negatively regulation of MDM2 alternative splicing by excluding exon 11. Other experiments in our lab showed that upon induction of stress, SC35 undergoes relocalization to larger and fewer nuclear speckles whereas SF2/ASF protein levels are increased. This allows us to proposed the working model for SC35 and SF2/ASF introduced in chapter 2. Consistent with the idea that misregulation of splicing factors and regulatory cis acting elements can promote the production of protein isoforms with roles in the emergence of disease including cancer, we believe that changes in the splicing regulation of MDM2 mediated by SC35 and SF2/ASF can lead to the generation of $MDM2$-$ALT1$.

We have generated a mouse model for the expression of $MDM2$-$ALT1$ in B cells. Our aim is to determine the role of $MDM2$-$ALT1$ in the emergence of B-cell malignancies. We demonstrate that our MDM2-$ALT1+/CD19{\text{Cre}+}$ mice express the transgene transcript in the spleen, where a high number of B cells is
expected. Monitoring of these mice for the development of solid tumors was carried out for a maximum period of two years and twenty one days. Although, no significant differences were found in survival and in tumorigenesis between the control transgene negative and MDM2-ALT1+ mice, other interesting significant findings emerged as a result of our investigation. Interestingly, these findings continue to enforce the dual role of MDM2-ALT1 in both cell proliferation inhibition and tumorigenesis. First, the spleen weight and the spleen:body weight ratio in the transgenic positive males appears to be significantly reduced in comparison to control transgene negative mice. It might be possible that this observation might be related to the inhibition of cell proliferation induced by the expression of MDM2-ALT1. Second, degenerative joint disease was significantly prevalent in MDM2-ALT1+ mice compared to control mice and this finding argues against the idea that MDM2-ALT1 inhibits cell proliferation. The clonal expansion of B cells in this inflammatory disease has been associated with mutations in the IgV<sub>H</sub> gene, which in turn are associated to the course of B-CLL. Strikingly, the reviewed literature proposes a relationship between the emergence of B-CLL, mutated and unmutated IgV<sub>H</sub> and dysfunctional p53. One proposed idea for the pathogenesis of B-CLL is that dysfunctional p53 renders the cells incapable of undergoing mutations in the IgV<sub>H</sub> that would result in a better B-CLL prognosis. If that is the case, it might be possible that MDM2-ALT1+ mice in which the degenerative joint disease was observed are under the protective period in which p53 is functional and capable to induced the hypermutations in the IgV<sub>H</sub> that are
characteristic of the clonal expansion of B cells observed in the inflammatory disease. Because p53 inactivation is central to our hypothesis the role of the MDM2-ALT1 in the emergence of degenerative joint disease might prove useful in our aim to elucidate the role of MDM2-ALT1 in tumorigenesis.

To effectively elucidate the role of this spliced form in tumorigenesis, an aggressive approach toward characterization of B cells expressing the transgene should be undertaken. Because our hypothesis is based on the interaction of full length MDM2 and MDM2-ALT1, it would be of utmost relevance to show the expression of this protein isoform in the B cells. We have aimed to do this in this study, but the protein isolation from splenic tissue after dedicating tissue for flow cytometry and pathological analysis, yielded low protein concentrations and western blot analysis are not conclusive at this time. This also limited the possibility of carrying western blot analysis targeting proteins that are involved in the p53 pathway (p53, p21, Bcl2 etc.) The lack of efficient antibodies targeting MDM2-ALT1 also contributes to the limitation to achieve this important aim. However, a flow cytometric approach to detect intracellular levels of p53 in B cells could be carried out to associate the expression of MDM2-ALT1 to the expression of p53 in B cells from transgene positive mice. Also, a BrdU incorporation assay looking at changes in proliferation upon the expression of MDM2-ALT1 in transgenic mice could bring relevant findings towards the role of MDM2-ALT1 in tumorigenesis. The timepoint at which these experiments are carried out would also be key in order to link a possible phenotype with the
persistent expression of the transgene and consequently to the induction of mutations by MDM2-ALT1. To achieve this sequencing of the p53 gene from tissue expressing the transgene should be carried out at different age timepoints. At the end, it might be possible that MDM2-ALT1 indeed has dual roles in both cell proliferation inhibition and tumorigenesis. Identifying the timepoint or the cellular context in which the protective period ends and tumorigenesis emerges could prove to be useful to therapeutically target this MDM2 protein isoform prior to the initiation of its tumorigenic function.
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