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ATM Phosphorylates and Activates the Transcription Factor MEF2D for Neuronal Survival in Response to DNA Damage

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Molecular Pathology

by

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ABSTRACT OF THE DISSERTATION

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Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by many clinical manifestations including neurodegeneration, most notably in the cerebellum resulting in gait ataxia and cancer predisposition. The mutated gene responsible for A-T is *ATM* (*ataxia telangiectasia mutated*), which encodes a kinase that activates multiple signal transduction pathways in response to DNA damage. Defects in survival signaling and the DNA damage response in neurons may cause the

neurodegenerative pathology of A-T, but ATM substrates in the central nervous system are as yet unclear.

Using protein sequence analysis, I have identified four potential ATM consensus phosphorylation motifs and one ATM-interaction motif in the neuronal pro-survival transcription factor, myocyte enhancer factor 2D (MEF2D). MEF2 represents a family of MADS (MCM1-agamous-deficiens-serum response factor) domain-containing transcription factors and plays a critical role in the nervous system regulating neurogenesis, synaptic plasticity and neuronal survival. There are four members of the MEF2 family, MEF2A to -D. MEF2A and -D predominate in the cerebellum, which is most affected in A-T. The activity of MEF2 proteins is governed in part by phosphorylation. Using *in vitro* immunocomplex kinase assays, I found that ATM phosphorylates MEF2A, -C and -D. DNA damaging agents that induce double-strand breaks increased phosphorylation of MEF2A and -D in cerebellar granule cell neurons. MEF2D phosphorylation was detectable in *Atm* wild-type cells but not in *Atm*-deficient cells. GAL4-dependent luciferase reporter gene assays revealed that ATM activates MEF2A and -D activity, but attenuates MEF2C activity. In addition, MEF2-dependent luciferase reporter gene assays showed that ATM increases endogenous MEF2 activity, and the potentiation of endogenous MEF2 activity is abolished either by RNA interference targeting ATM or by a small molecule inhibitor of ATM, KU-55933. Analysis by site-directed mutagenesis indicated that MEF2D is phosphorylated by ATM at four ATM consensus phosphorylation sites: Thr^{259}, Ser^{275}, Ser^{294} and Ser^{314}. Knockdown of endogenous MEF2D expression by a short-hairpin RNA (shRNA) increased cellular sensitivity to etoposide-induced neuronal cell death. Interestingly,

substitution of endogenous MEF2D expression with an shRNA-resistant phospho-mimetic MEF2D mutant protected primary neurons from cell death after DNA damage, whereas an shRNA-resistant nonphosphorylatable MEF2D mutant did not. Co-immunoprecipitation studies indicated that ATM and MEF2D form a complex following DNA damage. Collectively, these results suggest that ATM associates with MEF2D and activates its activity via phosphorylation, thus promoting neuronal survival in response to DNA damage.

Chapter 1: Ataxia-telangiectasia (A-T) and Ataxia-telangiectasia Mutated (ATM)

1.1. Clinical Features of Ataxia-telangiectasia (A-T)

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by a wide range of clinical manifestations including progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation (IR), premature aging, hypogonadism, growth retardation, immunodeficiency and increased risk of cancer (Jackson, 1995; Hoekstra, 1997; Gatti, 1998; Lavin *et al*., 2007). A-T occurs with a worldwide incidence of about 1 per 40,000 live births (Boder and Sedgwick, 1970; Lavin *et al*., 2007). A-T patients appear normal at birth, and the first signs of disease usually appear at the end of the first year of life (Gatti, 1998; Spacey *et al*., 2000; Lavin *et al*., 2007). At present, there is no cure for A-T. Clinical features of A-T are summarized in Table 1-1.

Table 1-1. A summary of clinical characteristics of A-T

Clinical features
Ataxia
Cerebellar degeneration
Ocular telangiectases
Choreoathetosis
Predisposition to cancer
Radiosensitivity
Immunodeficiency
Growth retardation
Elevated alpha-fetoprotein level

Neurodegeneration

The earliest clinical symptom and the pathological hallmark of A-T is ataxia (Crawford *et al*., 2000). A-T is marked by progressive impairment of gait and speech, oculomotor apraxia (an inability to move the eyes from side-to-side) and choreoathetosis (involuntary movements) (Gatti, 1998; Crawford *et al*., 2000; Spacey *et al*., 2000; Lavin *et al*., 2007). A-T patients are usually confined to a wheelchair by the end of the first decade of life (Lavin *et al*., 2007). The cause of the most debilitating symptoms of A-T is progressive neurodegeneration in the cerebellum. Purkinje cells and cerebellar granule cell neurons degenerate while adjacent basket cells (γ-aminobutyric acid-ergic (GABAergic) interneurons in the molecular layer) remain unaffected (Gatti, 1998; Spacey *et al*., 2000; Lavin *et al*., 2007). Degenerative changes in the cerebellar dentate and olivary nuclei, spinal cord and ganglia, cerebrum, basal ganglia and brain stem have also been described later in life (Gatti, 1998; Lavin *et al*., 2007).

Immunodeficiency and chromosomal instability

A-T is a primary immunodeficiency affecting both cellular and humoral immunity (Regueiro *et al*., 2000). B and T cells lineages are affected. The levels of immunoglobulins IgA and IgE are reduced, and abnormalities are observed in IgM and IgG (Regueiro *et al*., 2000). As a result, A-T patients show decreased resistance to common bacterial pathogens and viruses, and A-T patients are susceptible to intercurrent infections. Infections such as otitis, sinusitis and recurrent pneumonia can eventually lead to respiratory insufficiency and death (Sedgwick and Boder, 1991).

Defective development of the thymus is another characteristic of A-T (Regueiro *et al*., 2000). Hassell's corpuscles and corticomedullary demarcations are absent in A-T patients. A-T lymphocytes exhibit chromosomal instability mainly involving chromosomes 7 and 14 (Aurias *et al*., 1980). Chromosomal translocations of 7:14 near the T cell receptor loci often lead to T cell leukemia and lymphomas in A-T patients (Taylor *et al*., 1996; Liyanage *et al*., 2000). Translocations are not only restricted to chromosomal aberrations. Telomeric fusions and shortened telomeres are also found in A-T (Pandita *et al*., 1995; Metcalfe *et al*., 1996; Smilenov *et al*., 1997).

Cancer

Another manifestation of A-T is an increased incidence of neoplasms of the lymphoreticular system (Peterson *et al.*, 1992; Taylor, 1992). About 48% of A-T patients develop neoplasms during their lifetime (Peterson *et al.*, 1992; Taylor, 1992), of which T cell leukemia and lymphoma are the most common forms (Hecht and Hecht, 1990). Because A-T patients are exquisitely sensitive to IR, radiotherapy is avoided in the treatment of these neoplasms. There is also an increased incidence of cancers, especially breast cancer, in individuals heterozygous for the A-T mutation (Swift *et al*., 1991).

Other clinical features

Another major characteristic of A-T is telangiectasia (dilation of blood vessels), which appears in the corners of the eyes and sometimes in the facial skin and ears or under the tongue (McFarlin *et al*., 1972; Boder, 1985). Telangiectasia usually has a later onset than ataxia and occurs between 2 and 8 years of age. Elevated serum levels of

alpha-fetoprotein and carcinoembryonic antigens have also been described (Chun and Gatti, 2004).

1.2. Ataxia-telangiectasia Mutated (ATM) Function

The mutated gene responsible for A-T is *ATM* (ataxia-telangiectasia mutated). *ATM* was identified by positional cloning on chromosome 11q22-23 (Lange *et al*., 1995; Savitsky *et al*., 1995). It is composed of 66 exons, spanning 150 kb of genomic DNA and encodes an mRNA of 13 kb (Lange *et al*., 1995; Savitsky *et al*., 1995). The ATM mRNA is ubiquitously expressed in the brain, thymus, testis, ovary, heart, pancreas, spleen, kidney, skeletal muscle and liver (Savitsky *et al*., 1995). Mutations that cause A-T are spread throughout the *ATM* gene and consist of small to large deletions, insertions, missense mutations and single nucleotide changes, many of which may lead to splicing defects (Telatar *et al*., 1996; Wright *et al*., 1996; Stankovic *et al*., 1998; Castellví-Bel *et al*., 1999). These mutations may generate truncated proteins, resulting in a loss of function (Telatar *et al*., 1996; Wright *et al*., 1996; Stankovic *et al*., 1998; Castellví-Bel *et al*., 1999).

ATM encodes a 370-kDa protein that has a serine/threonine protein kinase activity. The carboxyl-terminal domain of ATM is similar to that of proteins in the phosphatidyl-inositol-3-kinase (PI3K) family (Savitsky *et al*., 1995; Rotman and Shiloh, 1998; Bakkenist and Kastan, 2003; Shiloh, 2003). ATM belongs to the PI3K-related kinase (PIKK) family. Proteins in the PIKK family include ATM and Rad3-related kinase (ATR), ATM-related kinase (ATX), target of rapamycin (mTOR),

transformation/transcription domain-associated protein (TRRAP) (a histone acetyltransferase) and DNA-dependent protein kinase (DNA-PK) (Abraham, 2001; Durocher and Jackson, 2001). Although PIKK proteins share the PI3K domain found in lipid kinases, these kinases only manifest protein kinase activity (Abraham, 2001; Durocher and Jackson, 2001).

ATM is an important transducer of the DNA damage signal and plays a critical role in the cellular response to DNA damage, activating DNA repair and cell cycle checkpoints, and coordinating the balance between cell survival and apoptosis (Khanna *et al*., 2001; Shiloh, 2003). ATM is activated by autophosphorylation at several sites in response to double-strand breaks (DSBs) in DNA (Bakkenist and Kastan, 2003; Kozlov *et al*., 2006). DSBs are the most severe form of DNA damage and are generated by exogenous agents such as IR or by endogenously generated reactive oxygen species (ROS) (Khanna and Jackson, 2001). DSBs also occur as intermediates during meiotic and V(D)J recombination (Khanna and Jackson, 2001). V(D)J recombination is the specialized DNA rearrangement used by cells of the immune system to assemble immunoglobulin and T-cell receptor genes from the preexisting gene segments.

In response to DSBs, cells stimulate DNA damage response (DDR) pathways, which involve activation of multiple signal transduction pathways to maintain genomic integrity and cell homeostasis. Activation of ATM depends on the presence of the MRE11/RAD50/NBS1 (MRN) complex, which acts as a DNA damage sensor. Once activated, ATM then phosphorylates a wide spectrum of substrates to mediate the response to DNA damage as shown in Figure 1-1.

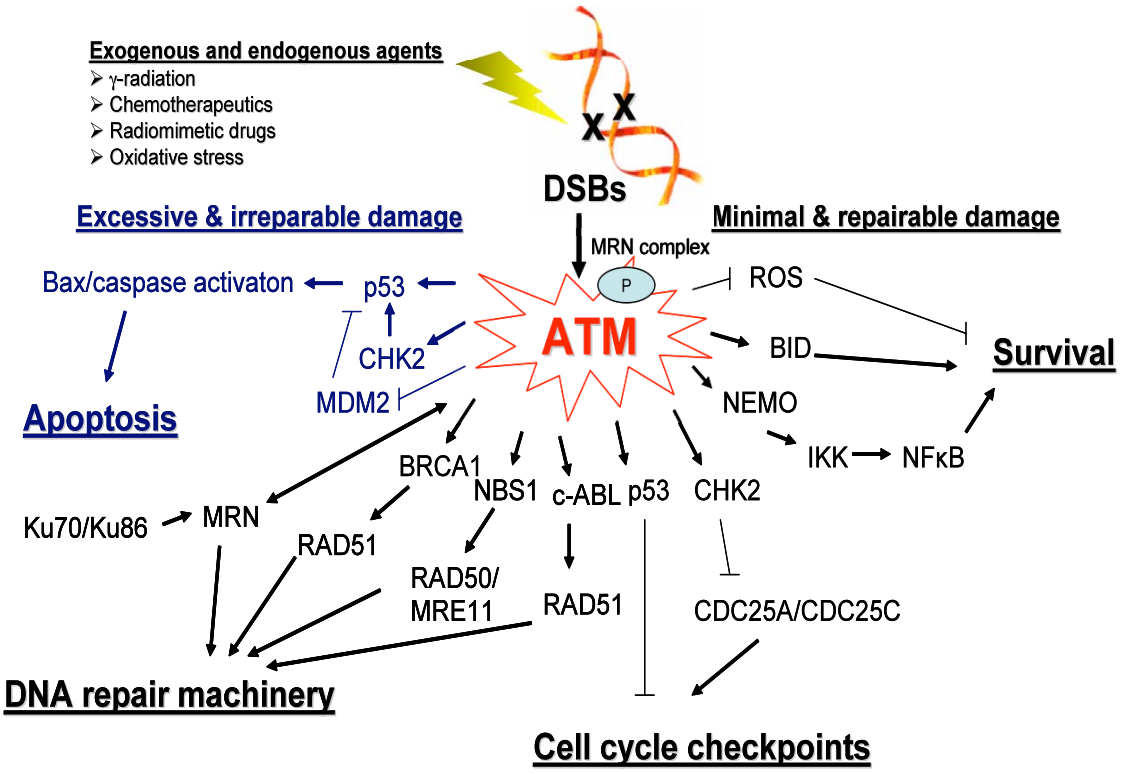

Figure 1-1. ATM-dependent DNA damage response pathway
Signaling in response to DNA double-strand breaks (DSBs) regulates a number of cellular processes, which include apoptosis, DNA repair machinery, cell cycle checkpoint activation and cell survival. Ataxia telangiectasia mutated (ATM) is activated through interaction with the DNA DSB sensor, the MRE11-RAD50-NBS1 (MRN) complex, which leads to autophosphorylation and activation of ATM. ATM then phosphorylates and thus regulates the activity of numerous downstream substrates to control the life and death of cells. In the face of excessive and irreparable DNA damage, apoptosis predominates. On the other hand, DNA repair machinery, cell cycle checkpoint activation and cell survival are initiated when DNA damage is minimal and repairable. ROS stands for reactive oxygen species.

ATM displays substrate specificity for serine or threonine residues that are followed by a glutamine (the SQ/TQ motif) (Kim *et al*., 1999; O'Neil *et al*., 2000). Hydrophobic amino acids and negatively charged amino acids adjacent to the amino terminal of the S/T residue are positive determinants for substrate phosphorylation, while positively charged amino acids are negative determinants (Kim *et al*., 1999; O'Neil *et al*., 2000). Biochemical and genetic analyses have identified numerous substrates for ATM,

and most are related to the regulation of cell cycle checkpoints and the development of cancer (Shiloh, 2003); however, the most prevalent feature of A-T is progressive neurodegeneration in the cerebellum. Little is known about ATM substrates in the central nervous system (CNS).

Cell cycle checkpoints

In response to DNA damage, ATM phosphorylates Ser 15 of the p53 tumor suppressor protein, which is a key regulator of cellular responses to genotoxic stress (Banin *et al*., 1998; Canman *et al*., 1998; Khanna *et al*., 1998). This serves to activate and stabilize p53 protein (Ashcroft *et al*., 1999; Dumaz and Meek, 1999). A rise in p53 protein levels induces the expression of p21 cyclin-dependent kinase inhibitor to prevent the normal progression from G1 to S phase, providing a check on replication of damaged DNA. The cell cycle is resumed after DNA repair is completed; however, if DNA damage is excessive and irreversible, apoptosis is initiated. There are also other ATM/p53-dependent regulators of the G1/S checkpoint. For instance, murine double minute 2 (MDM2) oncoprotein is a pivotal negative regulator of p53. ATM phosphorylates MDM2 at Ser 395, which blocks p53 translocation and stabilizes p53 levels (Khosravi *et al*., 1999; Maya *et al*., 2001). In contrast, p53 binding protein 1 (53BP1) is a p53 binding partner that can enhance the transcriptional activity of p53. ATM phosphorylates 53BP1 at several sites to activate p53-dependent transcription (DiTullio *et al*., 2002; Wang *et al*., 2002).

In the S-phase checkpoint, ATM regulates S-phase arrest after DNA damage by phosphorylating Nijmegen breakage syndrome 1 (NBS1) at Ser 343 and Ser 278. NBS1

is a key component of the MRN complex that is important for DSB repair and ATM activation (Gatei *et al*., 2000; Lim *et al*., 2000). In addition to NBS1, ATM also phosphorylates a protein named structural maintenance of chromosome 1 (SMC1) at Ser 957 and Ser 966. SMC1 participates in sister chromatid cohesion complexes and chromosome condensation, and its phosphorylation by ATM activates S-phase checkpoint in response to IR (Kim *et al*., 2002; Yazdi *et al*., 2002). Another ATM substrate is "breast cancer 1, early onset" (BRCA1) tumor suppressor protein, which acts as a regulator for both intra-S-phase and G2/M phase checkpoints. ATM phosphorylates and activates BRCA1 at several sites, including Ser 1387 for the initiation of S-phase arrest and Ser 1423 for G2/M phase transition (Cortez *et al*., 1999; Gatei *et al*., 2000; Gatei *et al*., 2001; Xu *et al*., 2001, 2002). Another key ATM substrate is Chk2 checkpoint kinase. ATM phosphorylates CHK2 at Thr 68, which in turn phosphorylates and degrades CDC25A protein (Bartek *et al*., 2001; Falck *et al*., 2001; McGowan, 2002). CDC25A is important for maintaining cyclin-dependent kinase 2 (CDK2) activity, which drives cells through the G1 to S transition and thus into S phase. CDC25A also regulates cyclin-dependent kinase 1 (CDK1) activity, which promotes the G2 to M transition. The degradation of CDC25A thus contributes to the progression of the G1/S, intra-S and G2/M checkpoints (Bartek *et al*., 2001; Falck *et al*., 2001; McGowan, 2002).

Apoptosis and cell survival

ATM also functions as a survival regulator to eliminate cells with excessive DNA damage. In the CNS, p53 is required for IR-induced apoptosis (Wood *et al*., 1995; Banin *et al*., 1998). The up-regulation of p53 after IR is absent or attenuated in ATM knockout

mice, resulting in resistance to apoptosis similar to that seen in p53 knockout mice (Banin *et al*., 1998; Canman *et al*., 1998; Lee and McKinnon, 2000). These findings suggest that ATM may activate p53 after IR to initiate the apoptotic signal cascade.

In contrast, ATM also acts in cells to promote survival pathways in the face of minimum and repairable DNA damage. ATM is activated by DNA damage, and this activation is required for cells to survive genotoxic stress. NF-kappa B, a pro-survival transcription factor in the proper context, modulates apoptotic responses induced by genotoxic stress (Hayden and Ghosh, 2004; Wu *et al*., 2006). ATM activates survival genes after the induction of DSBs, thereby preventing cell death, by phosphorylating and activating NF-kappa B essential modulator (NEMO) at Ser 85. NEMO is the regulatory subunit of IκB kinase (IKK), which phosphorylates NF-kappa B inhibitor IκB to prevent cell death (Wu *et al*., 2006). Interestingly, ATM phosphorylates BCL-2 interacting domain (BID), a protein that was previously considered to be a proapoptotic factor, at Ser 61 and Ser 78 to promote cell survival and S-phase arrest in response to DNA damage (Kamer *et al*., 2005; Zinkel *et al*., 2005). Both the ATM-NF-kappa B and ATM-BID signaling pathways are important for coordinating the DNA damage response and promoting cell survival. It will be interesting to determine whether there are similar pro-survival signaling cascades in the CNS.

Telomere and chromosome maintenance

ATM also plays a central role in maintaining telomere length and integrity (Zhang *et al*., 2007). The telomere is a region of repetitive DNA (TTAGGG) at the end of each chromosome. Together with several associated proteins, the telomere forms a

nucleoprotein complex to protect the chromosome ends from being recognized as DSBs and to prevent telomere erosion and end-to-end fusion of chromosomes. Proteins that stabilize telomeres include telomere repeat binding factor 1 (TRF1) and telomere repeat binding factor 2 (TRF2). TRF1 is a telomerase-specific binding protein that negatively regulates telomere elongation and affects mitotic progression. ATM binds and phosphorylates TRF1 at Ser 219 to suppress abortive mitosis and apoptosis and to initiate G2 arrest following DNA damage (Kishi *et al*., 2001). In A-T cells, inhibition of TRF1 rescues telomere shortening and decreases radiosensitivity. This finding provides additional evidence of a link between telomere metabolism and the DNA damage response. TRF2 protects telomeres through a different mechanism (Zhang *et al*., 2007), the formation of t-loop structures known as telosomes. Telosomes maintain telomere integrity by preventing chromosome ends from being recognized as DSBs (Zhang *et al*., 2007). TRF2 binds ATM, thereby inhibiting its activation at telomeres and suppressing ATM-dependent apoptosis (Karlseder *et al*., 2004). TRF2 is abundant at chromosome ends but not elsewhere in the nucleus. This checkpoint control mechanism can block the DDR at telomeres without affecting the surveillance of internal chromosome damage (Zhang *et al*., 2007).

A very early event in the cellular response to DSBs is the phosphorylation of a histone H2A variant, H2AX, at the site of DNA damage (Redon *et al*., 2002). ATM phosphorylates H2AX at Ser 139, resulting in chromosomal modification and initiation of the DNA repair process in response to DSBs (Burma *et al*., 2001).

Other ATM-dependent pathways

In response to DNA damage, ATM phosphorylates and activates c-ABL tyrosine kinase at Ser 465, which in turn induces tyrosine phosphorylation of RNA polymerase II (Baskaran *et al*., 1997; Shafman *et al*., 1997). This action may contribute to DNA damage-induced gene expression. After treatment of the cells with IR and hydrogen peroxide, ATM also phosphorylates Ca^{2+}/cAMP response element-binding protein (CREB), a transcription factor that regulates cell growth, homeostasis and survival (Shi *et al*., 2004). This phosphorylation of CREB decreases its activity and reduces its interaction with the transcriptional coactivator, CREB-binding protein (CBP) (Shi *et al*., 2004). However, the contribution of the ATM-CREB pathway to the neuropathogenesis of A-T remains unclear.

Additionally, ATM participates in cellular pathways that are not directly linked to the DNA damage response (Yang and Kastan, 2000; Bakkenist and Kastan, 2003; Fernandes *et al*., 2007). For example, compounds that change chromatin structure, such as trichostatin A or chloroquine, can activate ATM independently of DNA damage (Bakkenist and Kastan, 2003). Moreover, insulin resistance, glucose intolerance or reduction in IGF-1 receptor affinity have been observed in some A-T patients (Blevins and Gebhart, 1996). During insulin signaling, ATM is activated and phosphorylates eIF-4E-binding protein 1 **(**4E-BP1), a regulator of protein synthesis, at Ser 111 to induce the release of eukaryotic translation initiation factor 4E (eIF-4E). In turn, eIF-4E can enhance the initiation of mRNA translation to foster protein synthesis (Yang and Kastan, 2000). Although ATM may have some role in insulin signaling pathways, its actual function in these pathways remains to be explored.

ATM also manifests a novel role in promoting differentiation of SY5Y neuroblastoma cells into neuronal-like cells (Fernandes *et al*., 2007). In this case, retinoic acid (RA) activates ATM, which in turn phosphorylates CREB to induce extension of neuritic processes and differentiation of SH-SY5Y cells into neuronal-like cells (Fernandes *et al*., 2007). However, as noted above, ATM can also phosphorylate and inactivate CREB activity in response to IR or hydrogen peroxide (Shi *et al*., 2004). Thus, ATM may play a dual role in regulating CREB activity. CREB activity is increased in neuronal cells in response to growth signaling from RA, but decreased after DNA damage.

Biochemical and genetic analyses have identified numerous substrates for ATM (Shiloh, 2003), and the substrate list continues to expand (Matsuoka *et al*., 2007; Stokes *et al*., 2007). The absence of ATM gives rise to various disease phenotypes in mice and humans because ATM affects numerous cellular processes. Several ATM substrates may work cooperatively in the regulation of cell survival, apoptosis and DNA damage response pathways to account for some of the abnormalities seen in A-T cells.

Studies of ATM knockout mouse models of neurodegeneration

Inactivation of ATM in several different mouse models has provided valuable information about human A-T. These mice recapitulate many characteristics of human A-T, including growth retardation, radiosensitivity, immunodeficiency, predisposition to cancer and infertility (Barlow *et al*., 1996; Liyanage *et al*., 2000; Lavin *et al*., 2007). However, none of the mouse models show progressive neurodegeneration and ataxia similar to that found in human A-T. Some neurological abnormalities that have been

identified in these mice include impaired rotarod performance, reduction in the number of tyrosine hydroxylase-positive nigrostriatal neurons, abnormal changes in Purkinje cell dendritic arborization but no gross cerebellar pathology, absence of radiation-induced apoptosis in the CNS, and defective neural fate determination in the dentate gyrus of the hippocampal region of the adult (Barlow *et al*., 1996; Lavin *et al*., 2007).

Oxidative stress in neurons, leading to accumulation of DNA damage, has been associated with many neurodegenerative diseases (Rolig and McKinnon, 2000; Abner and McKinnon, 2004). Normally, this DNA damage would activate ATM, possibly to initiate repair and thus relieve the free radical stress. Elevated oxidative stress has been found in neurons in ATM knockout mice (Barlow *et al*., 1999; Peter *et al*., 2001; Chen *et al*., 2003). These mice have reduced catalase activity, increased superoxide dismutase (SOD) activity, increased hemoxygenase activity and increased production of ROS in the CNS (Barlow *et al*., 1999; Peter *et al*., 2001; Chen *et al*., 2003). It has been postulated that these abnormalities may contribute in part to the neurodegeneration observed in A-T.

Concerning the potential effect of ATM on cell cycle kinases, it is known that aberrant cell cycle entry in post-mitotic neurons leads to cell death rather than cell division (Love, 2003; Nguyen *et al*., 2003; Yang *et al*., 2003). Cell cycle proteins that are normally not expressed in post-mitotic neurons reappear in many neurodegenerative diseases such as stroke, amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Busser *et al*., 1998; Ranganathan *et al*., 2001; Jordan-Sciutto *et al*., 2003; Love, 2003; Nguyen *et al*., 2003; Yang *et al*., 2003). Cell cycle proteins are re-expressed in Purkinje cells, cerebellar granule cell neurons and striatal neurons in both human and mouse A-T (Yang and Herrup, 2005). These proteins include cyclin A, a S/G2-phase

marker; cyclin B, a regulatory subunit of cdc2 kinase found in the G2-phase; and proliferating cell nuclear antigen (PCNA), a subunit of the DNA polymerase holoenzyme that is elevated mainly during S-phase (Yang and Herrup, 2005). The contribution of these cell cycle events to A-T remains unknown.

1.3. Hypothesis and Specific Aims

As discussed above, it is well-established that ATM is a master regulator of the pathways that repair DNA damage, regulate cell cycle checkpoints, and coordinate the balance between cell survival and apoptosis in response to DNA damage. In the presence of repairable/minimal DNA damage, cells activate ATM-dependent DNA damage response pathways to maintain genomic integrity and cell homeostasis, thus promoting cell survival, initiating DNA damage repair and inducing cell cycle checkpoints. ATM-mediated activation of pro-survival genes may provide a window of opportunity for DNA repair pathways to correct the damage, allowing cells to return to their normal physiological state after DNA damage and survive the insult (Hayden and Ghosh, 2004; Wu *et al*., 2006).

On the other hand, cells may initiate apoptotic machinery in the face of extensive /irreparable DNA damage (Brooks, 2002; Kaina, 2003; Schwartz *et al*., 2007; Frappart and McKinnon, 2008). In fact, DNA damage has been associated with many neurodegenerative diseases (Rolig and McKinnon, 2000; Abner and McKinnon, 2004). Defects in the DNA damage response and survival signaling in neurons could potentially contribute to the neurodegenerative pathology of A-T. Understanding the neuronal

degeneration in A-T will require the elucidation of ATM function in neurons. The consensus phosphorylation motif for ATM is a serine or threonine residue followed by glutamine (the SQ/TQ motif). Numerous substrates for ATM have been identified, and most are related to the regulation of cell cycle checkpoints and development of cancer (Shiloh, 2003). However, ATM substrates in the CNS are as yet unclear. Using protein sequence analysis, I have identified four potential ATM consensus phosphorylation motifs and one ATM-interaction motif in the neuronal pro-survival transcription factor, myocyte enhancer factor-2D (MEF2D) (Figure 1-2). MEF2 represents a family of MADS (MCM1-agamous-deficiens-serum response factor) domain-containing transcription factors thought to play a critical role in the regulation of neurogenesis, synaptic plasticity, and neuronal survival during development of the CNS. Our group was the first to clone one of the MEF2 genes in the brain and the first to report their potential function in the CNS (Leifer *et al*., 1993; Okamoto *et al*., 2000, 2002). Of note, two of the MEF2 genes, MEF2A and -D, predominate in the cerebellum which is most affected in A-T. A detailed introduction to the MEF2 gene family and characterization of MEF2 function are elaborated in Chapter 2. For this dissertation work, I hypothesized that ATM phosphorylates and thereby activates MEF2D to promote survival of cerebellar granule cell neurons in response to DNA damage. These events may provide an opportunity for neurons to initiate antiapoptotic signaling and the DNA damage repair pathways in order to survive the insult and maintain the homeostasis of the nervous system. Thus, dysregulation of this ATM-MEF2D pathway may contribute to the neuropathogenesis in A-T.

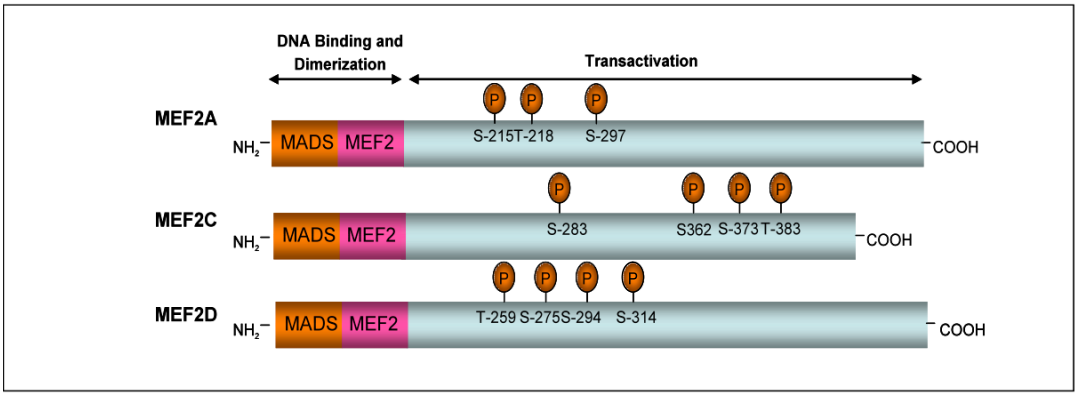

Figure 1-2. Potential ATM-phosphorylatable sites in MEF2.
Myocyte enhancer factor 2 (MEF2) contains a conserved N-terminal DNA binding and dimerization domain, and a C-terminal transactivation domain. Potential ATM-phosphorylatable sites in MEF2 have been identified in the transactivation domain and are marked with a "P" in the schematic.

To test the above hypothesis, I proposed the following Specific Aims during the course of this study:

1). To determine whether ATM phosphorylates MEF2 using two independent assays:

a). Detection of MEF2 phosphorylation *in vitro* using purified recombinant MEF2 fusion proteins in immunocomplex ATM kinase assay.

b). Detection of MEF2 phosphorylation in wild-type (ATM-positive) vs. ATM-null cells after DNA damage using Western blotting.

2). To examine whether ATM regulates MEF2 activity using two reporter gene assays, a GAL4-dependent luciferase reporter and a MEF2-dependent luciferase reporter under three different conditions:

a). Overexpression of wild-type or kinase-dead ATM in mammalian cells;

b). Knockdown of ATM expression using RNA interference in cerebellar granule cell neurons;

c). Inhibition of ATM kinase activity in intact cells using a small molecule inhibitor of ATM, KU-55933.

3). To map ATM-phosphorylatable sites in MEF2D using site-directed mutagenesis and to evaluate which ATM-phosphorylatable sites in MEF2D are essential for activation using reporter gene assays.

4). To investigate the physiological effect of ATM-mediated phosphorylation/activation of MEF2D on neuronal survival after DNA damage using a strategy (a combination of RNA interference and overexpression of short-hairpin RNA (shRNA) resistant MEF2D mutants) that assesses cerebellar granule cell viability after --

a). Inhibition of endogenous MEF2D expression using an shRNA targeting MEF2D, and;

b). Substitution of endogenous MEF2D with an shRNA-resistant --

i). wild-type MEF2D,

ii). phospho-mimetic MEF2D mutant, or

iii). nonphosphorylatable MEF2D mutant.

5). To determine whether ATM interacts with MEF2D using co-immunoprecipitation techniques.

Chapter 2: Myocyte Enhancer Factor 2 (MEF2)

2.1. Myocyte Enhancer Factor 2 (MEF2) Family of Transcription Factors

Myocyte enhance factor 2 (MEF2) represents a family of MADS domain-containing transcription factors that are thought to play a critical role in the regulation of many cellular processes such as cell differentiation, proliferation, morphogenesis, survival and apoptosis in a wide range of cell types (Mao *et al*., 1999; Okamoto *et al*., 2000; Li *et al*., 2001; Gaudilliere *et al*., 2002; Flavell *et al*., 2006; Shalizi *et al*., 2006). MEF2 is highly enriched in muscles and was first described as a transcription factor that binds to A/T-rich DNA sequences and regulates muscle-specific genes during myogenic development (Molkentin and Olson, 1996; Ornatsky *et al*., 1999). MEF2 cooperates with myogenic basic helix-loop-helix transcription factors such as MyoD to initiate myogenesis. Virtually at the same time as the muscle work was first published, our laboratory reported that MEF2 was present in the nervous system during neuronal differentiation (Leifer *et al*., 1993). MEF2 was subsequently found to be expressed at high levels in neurons and is a key determinant of neuronal fate, survival, differentiation and synaptic plasticity (Flavell and Greenberg, 2008).

There are four members of the MEF2 family, MEF2A to -D, that bind as homo- and heterodimers to the DNA consensus sequence $CTA(A/T)_4TA(G/A)$ (Yu *et al*., 1992; Leifer *et al*., 1993; Martin *et al*., 1994; Naya and Olson, 1999). Each MEF2 isoform exhibits distinct expression patterns in different regions of the brain (Leifer *et al*., 1993;

Ikeshima *et al*., 1995; Lyons *et al*., 1995; Lin *et al*., 1996). MEF2 expression is observed in neural crest as early as embryonic day (E) 8.5 and in the developing brain from E12.5 onward (Edmondson *et al*., 1994). All four MEF2 proteins are expressed in the cerebral cortex and olfactory bulb. MEF2A and -D express predominately in the cerebellum, which is most affected in A-T. MEF2D transcripts are detected throughout the developing CNS and into adulthood, while MEF2A transcripts are found in hippocampus, thalamus and internal granular layer of cerebellum (Lyons *et al*., 1995). MEF2B expression is similar to MEF2A in the brain. MEF2C protein is observed in a subset of cortical neurons in layers II, IV and VI (Leifer *et al*., 1993; McDermott *et al*., 1993).

MEF2 isoforms share homology in an amino-terminal 56 amino acid MADS domain, and an adjacent 29 amino acid MEF2 domain, which together mediate DNA binding and dimerization. The sequences outside these two domains, the carboxyl-terminal of MEF2, consists of a transactivation domain which is required for transcriptional activation (McKinsey *et al*., 2002) and is relatively divergent among MEF2 isoforms (Yu *et al*., 1992; Leifer *et al*., 1993; Martin *et al*., 1994; Naya and Olson, 1999) (Figure 2-1).

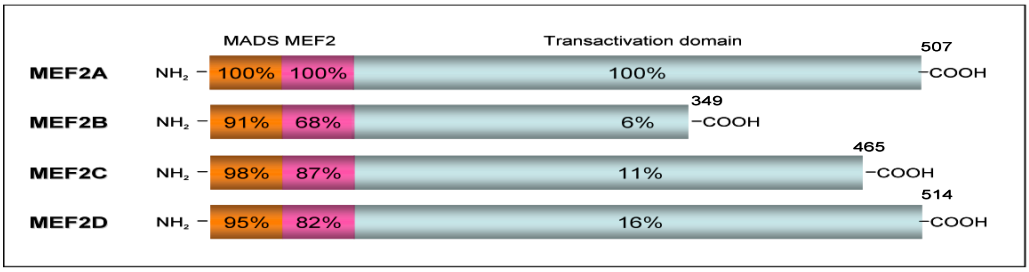

Figure 2-1. MEF2 amino acid sequence identity.
Percentage of amino acid identity within the MADS, MEF2 and transactivation domains of each MEF2 family protein is shown in relative to MEF2A, which is set to 100%. The number of amino acids in each MEF2 protein is indicated at the carboxyl-terminal end.

2.2. MEF2 Functions and Signaling Cascades in the CNS

MEF2 is pivotal for mediating survival, synapse formation, dendritogenesis, differentiation and lineage specification in neurons (Okamoto *et al*., 2000; Flavell and Greenberg, 2008; Fiore *et al*., 2009). MEF2 function is regulated by post-translational modifications including phosphorylation, acetylation and sumolyation (Black and Olson, 1998; Shalizi *et al*., 2006; Flavell and Greenberg, 2008). Many signal transduction pathways promote MEF2 phosphorylation and modulate the pro-survival function of MEF2 in the CNS. Several kinases including p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 5 (ERK5) and cyclin-dependent kinase 5 (CDK5) have been shown to phosphorylate MEF2 and regulate MEF2 activity (Figure 2-2). Calcium signaling plays a key role in eliciting neuronal activity-dependent transcription. In response to calcium influx following depolarization, p38 MAPK activates and phosphorylates MEF2 to promote neuronal survival in cerebellar granule cell neurons and neuronally differentiated P19 embryonic carcinoma precursor cells (Mao *et al*., 1999; Okamoto *et al*., 2000). Moreover, brain-derived neurotrophic factor (BDNF) promotes neuronal survival through activation of ERK5, which in turn phosphorylates and activates MEF2 activity to induce neurotrophin NT-3 expression (Shalizi *et al*., 2003) (Figure 2-2).

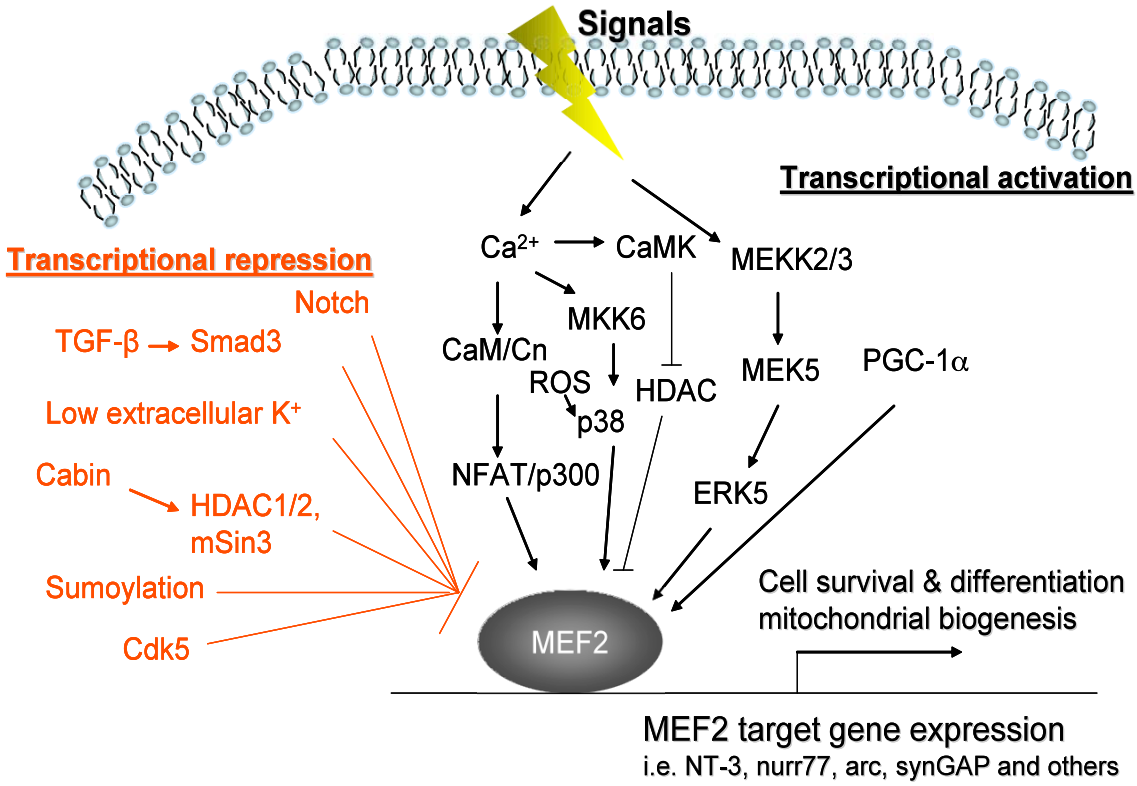

Figure 2-2. Regulation of MEF2 signaling cascade.
MEF2 acts as a transcriptional effector of numerous signaling pathways to regulate many cellular processes including cell survival, differentiation and mitochondrial biogenesis. Many post-translational modifications and multiple MEF2-interacting proteins have been identified to control MEF2 function and its downstream target genes. Some of the cofactors stimulate MEF2 activity (activators) and others repress MEF2 function (repressors).

In contrast, CDK5 can phosphorylate MEF2 when activated in response to excitotoxic insults or oxidative stress and inhibit MEF2 activity to induce neuronal apoptosis in cerebellar granule cell neurons (Gong *et al*., 2003) (Figure 2-2). In the absence of depolarizing potassium, MEF2A and -D are hyperphosphorylated and targeted for caspase-mediated cleavage in cerebellar granule cell neurons (Li *et al*., 2001) (Figure 2-2). Caspase-mediated cleavage of MEF2A or -D generates two fragments, one containing the transactivation domain and another one containing the MADS and MEF2 domain that can act as dominant-negative to induce apoptotic cell death (Li *et al*., 2001).

However, the kinase that induces MEF2A and -D hyperphosphorylation is still unclear. Exposure of cortical neurons to excitotoxic concentrations of *N*-methyl-D-aspartic acid (NMDA) can also induce caspase-mediated cleavage of various MEF2 isoforms, as can cerebral ischemia *in vivo*, thus generating dominant-negative of MEF2 to induce apoptosis (Okamoto *et al*., 2002). In addition, knockdown of MEF2A expression using RNA interference (RNAi) in primary cerebellar cultures markedly decreases cerebellar granule cell neuronal survival (Gaudilliere *et al*., 2002).

MEF2 also plays a central role in regulating synapse formation and postsynaptic differentiation of dendrites (Flavell *et al*., 2006; Shalizi *et al*., 2006; Fiore *et al*., 2009). In the absence of calcium influx, MEF2A is sumoylated at Lys 403 and acts as a transcriptional repressor to suppress the expression of the NUR77 transcription factor, a negative regulator of dendritic differentiation (Scheschonka *et al*., 2007) (Figure 2-2). This repression of NUR77 promotes dendritic claw differentiation (Shalizi *et al*., 2006). On the other hand, calcium influx triggered by neuronal activity causes MEF2A dephosphorylation at Ser 408 by calcineurin, a serine/threonine phosphatase that is activated by calcium signals. This promotes desumoylation and acetylation of MEF2A at Lys 403 to restrict synapse formation of hippocampal neurons through the induction of the activity-regulated cytoskeletal-associated protein (arc) and synaptic Ras guanosine triphosphatase activating protein (synGAP) expression (Flavell *et al*., 2006). Additionally, MEF2A activation inhibits postsynaptic differentiation of dendrites in the cerebellum (Shalizi *et al*., 2006). Another study showed that cocaine exposure increases MEF2 phosphorylation at its inhibitory CDK5 site and suppresses MEF2 activity to

promote dendritic spine density on medium-sized spine neurons in the nucleus accumbens (Pulipparacharuvil *et al.*, 2008).

In contrast, a recent study reported that MEF2 acts as a positive regulator of dendritogenesis (Fiore *et al*., 2009). In this case, MEF2 induces expression of microRNA-134 (miRNA-134), which inhibits the translation of mRNA encoding the RNA binding protein and translational repressor Pumilio 2, and therefore promotes dendritic outgrowth. A lower level of Pumilio 2 allows the translation of a set of unknown mRNAs that are likely to be positive regulators of dendrite development (Fiore *et al*., 2009). Consistent with these opposing effects, known MEF2 targets include both dendrite growth-promoting factors, such as BDNF, and negative regulators of synapse formation such as arc and synGAP (Flavell and Greenberg, 2008). This suggests that MEF2 has dual functions and might regulate different sets of target genes in different contexts and conditions of neuronal development.

MEF2 regulates neuronal development not only by promoting survival and differentiation but also by directing P19 embryonal carcinoma cells and murine embryonic stem cells (ESCs) towards neuronal lineage specification (Okamoto *et al*., 2000; Skerjance and Wilton, 2000; Li *et al*., 2008; Schneider *et al*., 2008). A neurogenic basic helix-loop-helix (bHLH) protein, mammalian achaete-scute homologs 1 (MASH1), which is essential for neuronal fate determination, interacts directly with MEF2 to synergistically activate transcription in neurogenic cell lineages (Black *et al*., 1996; Skerjance and Wilton, 2000). Following treatment with retinoic acid (RA), MEF2A and -C, but not -D are up-regulated during neural differentiation in P19 cells. Recently, our group used a constitutively active form of MEF2C (MEF2CA), which encodes the DNA-

binding and dimerization domain of MEF2 fused to the VP16 activation domain under the regulation of the nestin enhancer to drive the differentiation of ESCs into neuronal progenitor cells (Li *et al*., 2008). These cells display functional and electrophysiological characteristics of neurons including tetrodotoxin-sensitive sodium currents and GABA- or glutamate-evoked currents (Li *et al*., 2008).

2.3. MEF2 Functions and Signaling Cascades Outside of the CNS

In addition to its effects on neurons, MEF2 plays a key role in many cellular processes in various cell types such as skeletal muscle cells, cardiac myocytes and T cells. MEF2 has been most extensively studied in muscle cells (Black *et al*., 1998). MEF2 is highly enriched in muscles and was initially described as a transcription factor to regulate muscle-specific genes during myogenic development. MEF2 activity is regulated by calcium signaling and direct physical interactions with a large collection of cofactors such as MyoD, Notch, GATA, thyroid hormone (TH) receptor, the nuclear factor of activated T cells (NFAT), p300 and histone deacetylases (HDACs) (Molkentin *et al*., 1995; Lee *et al*., 1997; Sartorelli *et al*., 1997; Miska *et al*., 1999; Wilson-Rawls *et al*., 1999; Blaeser *et al*., 2000; Morin *et al*., 2000; Youn *et al*., 2000) (Figure 2-2). MEF2 alone does not possess myogenic activity but cooperates with myogenic bHLH transcription factors such as MyoD or myogenin to activate myogenesis (Molkentin and Olson, 1996; Ornatsky *et al*., 1999; Wang *et al*., 2001). Additionally, MEF2 interacts with Notch, a transmembrane receptor that controls cell fate decisions and patterned differentiation in numerous developmental processes (Artavanis-Tsakonas *et al*., 1999;

Kopan, 2002). In this context, myogenic activity is actually inhibited (Wilson-Rawls *et al*., 1999). To account for this finding, transforming growth factor-β (TGF-β) is known to inhibit myogenesis and associated gene expression (Brennan *et al*., 1991). TGF-β activates its effector, Smad3, which then interacts with MEF2C and subsequently inhibits its transcriptional activity to repress myogenic differentiation (Liu *et al*., 2004).

MEF2 is also a key regulator for cardiac development and various pathophysiologic processes (Han and Molkentin, 2000). MEF2 associates with the GATA-4 cardiac-specific transcription factor or with the TH receptor in adult cardiomyocytes to regulate cardiac gene expression involved in cardiac myogenesis (Lee *et al*., 1997; Morin *et al*., 2000). Phosphorylation of MEF2 by p38 MAPK can stimulate skeletal muscle differentiation and mediate pathologic cardiomyocyte hypertrophy (Kolodziejczyk *et al*., 1999; Zetser *et al*., 1999; Wu and Olson, 2002).

MEF2 also interacts with repressors, such as the class II HDACs, HDAC4, -5, -7 and -9, which leads to deacetylation of nucleosomal histones enclosing MEF2 DNA-binding sites and suppression of MEF2-dependent genes (Grozinger and Schreiber, 2000; McKinsey *et al*., 2000; Wang *et al*., 2000; Wang and Yang, 2001; McKinsey and Olson, 2005).

MEF2 is thought to be a calcium-dependent regulator of many cellular processes such as cell division and differentiation (McKinsey *et al*., 2002). Calcium/calmodulin-dependent protein kinase (CaMK) phosphorylates HDACs and subsequently creates docking sites for the chaperone protein 14-3-3. After binding to 14-3-3, it promotes nuclear-to-cytoplasmic shuttling of HDACs, and HDACs are subsequently released from MEF2. MEF2 is then bound by the p300 coactivator, which has histone acetyltransferase

(HAT) activity; this stimulates MEF2 activity and MEF2 target gene transcription (Grozinger and Schreiber, 2000; McKinsey *et al*., 2000; Wang *et al*., 2000; Wang and Yang, 2001; McKinsey and Olson, 2005).

In addition, in response to a rise in intracellular calcium, calcineurin, a serine/threonine phosphatase is activated by binding of the calcium-binding protein calmodulin (CaM), and subsequently dephosphorylates the NFAT transcription factor (McKinsey *et al*., 2002). After NFAT is dephosphorylated, it associates with MEF2 and recruits the p300 coactivator to stimulate MEF2 activity.

In T lymphocytes, MEF2 associates with the repressor, cabin, which recruits the class I HDACs, HDAC1 and -2 through the mSin3 co-repressor (McKinsey *et al*., 2002) (Figure 2-2). This leads to deacetylation of histones and repression of MEF2 target gene transcription. An elevation of intracellular calcium triggers the release of the co-repressor complexes and promotes the association of MEF2 with NFAT and p300 to activate MEF2 target gene expression (McKinsey *et al*., 2002).

MEF2 not only regulates muscle development and calcium-dependent gene expression but also controls mitochondrial biogenesis in the heart. Proliferator-activated receptor-γ (PPAR-γ) coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis and energy homeostasis (Puigserver and Spiegelman, 2003). Overexpression of PGC-1α in the heart increases mitochondrial biogenesis (Lehman *et al*., 2000). In contrast, downregulation of PGC-1α expression results in a loss of cardiac mitochondria (Czubryt *et al*., 2003). MEF2 acts as a coactivator of PGC-1α and also stimulates PGC-1α gene expression (Wu *et al*., 2002; Czubryt *et al*., 2003; Handschin *et al.*, 2003) (Figure 2-2). The interaction with PGC-1α may be essential for MEF2A function

because MEF2A knockout mice show a profound disruption of mitochondrial organization and gene expression (Naya *et al*., 2002).

MEF2 appears to be required in the neural crest not only for neuronal differentiation but also for craniofacial development (Verzi *et al*., 2007). Loss of MEF2C in neural crest cells results in neonatal lethality because of severe craniofacial defects (Verzi *et al*., 2007). MEF2C activates the expression of the distal-less homeobox 5 and -6 (DLX5 and -6) transcription factors and acts synergistically to direct craniofacial development (Miller *et al*., 2007; Verzi *et al*., 2007).

MicroRNAs are thought to have critical roles in diverse biological processes such as muscle development and progenitor cell proliferation (Bartel, 2004; Taganov *et al*., 2007; van Rooij *et al*., 2008). Many muscle-specific microRNAs have been shown to regulate diverse aspects of muscle function such as myoblast proliferation and differentiation (van Rooij *et al*., 2008). An intragenic MEF2-dependent enhancer controls muscle-specific expression of microRNA 1 (miR-1) to promote myoblast differentiation by repressing HDAC4. MEF2 also regulates microRNA 133 (miR-133) expressions to enhance myoblast proliferation by repressing serum response factor (SRF) expression, an essential activator of myogenesis. Recently, another study suggested that MEF2C promotes myeloid progenitor proliferation (Johnnidis *et al*., 2008). MEF2C is a functional target of myeloid-specific miR-223, which negatively regulates progenitor proliferation and granulocyte differentiation and activation. miR-223-null mice have an expanded granulocytic compartment, and this phenotype can be rescued by genetic ablation of MEF2C. These results suggest that miR-223 and MEF2C play a key role in regulating granulocyte production (Johnnidis *et al*., 2008).

2.4. Studies of MEF2 Knockout Mice

Loss-of-function studies in mouse models have revealed distinct functions for MEF2A, -C and -D in heart development. MEF2A knockout mice are highly susceptible to cardiac arrest (Naya *et al*., 2002). Most die within the first week of life. MEF2A mice display dilation of the right ventricle of the heart, fragmentation of myofibrils and mitochondrial structural abnormalities (Naya *et al*., 2002). Mice that survive to adulthood also have a shortage of cardiac mitochondria and are susceptible to sudden death (Naya *et al*., 2002). These findings suggest that MEF2A is important for maintaining mitochondrial content and architectural integrity in the postnatal heart. On the other hand, MEF2D knockout mice are viable and show resistance to develop cardiac hypertrophy, fibrosis and activate fetal cardiac gene expression in response to stress signals such as pressure overload and chronic β-adrenergic stimulation (Kim *et al*., 2008). In a MEF2D transgenic mouse model, overexpression of MEF2D drives fetal cardiac gene program and pathological remodeling of the heart (Kim *et al*., 2008). These results reveal that MEF2D is important for mediating stress-dependent cardiac growth and reprogramming of gene expression in adult heart. The brain function of MEF2D in these mice has not been explored yet. MEF2C knockout mice die embryonically due to defective cardiac myogenesis and morphogenesis (Lin *et al*, 1997). To better understand MEF2 functions in the CNS, our group has recently generated conditional knockout MEF2C mice by crossing floxed MEF2C mice with Nestin-*Cre* mice (Li *et al*., 2008). Using this strategy, MEF2C is conditionally knocked out in nestin-expressing neural stem/progenitor cells (Li *et al*., 2008). MEF2C conditional knockout mice are viable but

have a smaller whole brain size, and fewer neurons and synapses. Knockout of MEF2C in neural progenitors causes abnormal aggregation and compaction of neurons that migrate into the lower layers of neocortex during CNS development (Li *et al*., 2008). MEF2C conditional knockout mice show behavioral deficits such as abnormal anxiety-like behaviors, low levels of object exploration and marked paw wringing/clasping stereotypy, which are reminiscent of Rett syndrome, an autism-spectrum disorder (Li *et al*., 2008).

Chapter 3: Results

DNA damage has been linked with many neurodegenerative diseases (Rolig and McKinnon, 2000; Abner and McKinnon, 2004). Defects in the DNA damage response and survival signaling in neurons may result in neurodegeneration in A-T. Understanding the neurodegeneration in A-T will require exploring ATM functions in the brain. Neuronal pro-survival transcription factor MEF2D contains four potential ATM consensus phosphorylation motifs and one ATM-interaction motif (Figure 1-2). I hypothesize that ATM phosphorylates MEF2D, and subsequently stimulates MEF2D survival activity to prevent cell death in cerebellar granule cell neurons after DNA damage. The activation of ATM-MEF2D signaling event may provide an opportunity for neurons to initiate survival signaling in order to survive the insult, maintain cell homeostasis and enable neurons to return to their normal physiological state after DNA damage.

3.1. ATM Phosphorylates MEF2 *In Vitro*

I first used i*n vitro* immunocomplex kinase assays to determine whether ATM phosphorylates MEF2. Cell extracts from human embryonic kidney (HEK) 293T cells that had been transfected with cDNAs encoding Flag-tagged wild-type (w.t., catalytic active) or kinase-dead (k.d., dominant negative) ATM (Bakkenist and Kastan, 2003) were immunoprecipitated using anti-Flag antibody. The immunoprecipitants were then

incubated with recombinant GST-p53 or His-tagged MEF2A, -C or -D fusion proteins and [γ-^{32}P] ATP. The ^{32}P-labeled proteins were detected among the His-tagged MEF2A,-C and -D in the presence of w.t. ATM but not k.d. ATM (Figure 3-1). Phosphorylation of GST-p53 by w.t. ATM was used a positive control. These results show that ATM phosphorylates MEF2 *in vitro*.

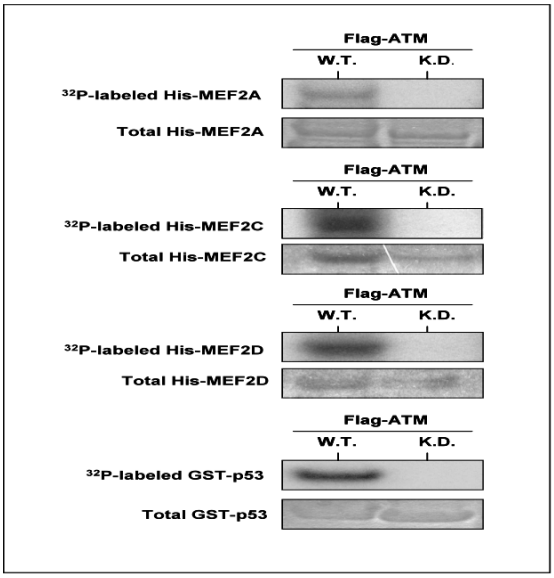

Figure 3-1. ATM phosphorylates MEF2 *in vitro*.
Cell extracts from human embryonic kidney (HEK) 293T cells that had been transfected with 10 μg of wild-type (w.t.) or kinase-dead (k.d.) ATM cDNAs were immunoprecipitated with anti-Flag M2 antibody (Sigma) and protein A/G-agarose. The immunoprecipitants were then incubated with 10 μCi of [γ-32P] ATP and 1 μg of recombinant GST-p53 or the indicated His-tagged MEF2A or -C or -D fusion proteins. The kinase reaction was conducted at 30 ^{o}C for 20 min and stopped by the addition of SDS-PAGE loading buffer. Proteins were separated with SDS-PAGE, and radiolabeled proteins were assessed by autoradiography. Total protein loading levels were determined by Coomassie brilliant blue staining.

3.2. DNA Damaging Agents That Induce Double-strand Breaks (DSBs) Trigger ATM-mediated Phosphorylation of MEF2

Next, I explored whether ATM phosphorylates MEF2 in cerebellar granule cell neurons in response to DNA damage. Primary cerebellar granule cell neurons were used in this study because they are one of the cell types most affected in A-T. Cerebellar granule cell neurons were first treated with DNA damaging agents, IR or the topoisomerase II inhibitor etoposide, which can induce DSBs in DNA. Cell extracts were then immunoprecipitated with either a control anti-IgG, an anti-MEF2A, or an anti-MEF2D antibody. ATM-phosphorylated MEF2A and -D proteins were detected by immunoblotting with an anti-phospho-SQ/TQ substrate antibody, which can detect phosphorylated ATM substrates. IR and etoposide increased ATM-phosphorylated MEF2A and -D protein levels (Figure 3-2).

To verify this phosphorylation further, cerebellar granule cell neurons were first treated with IR or etoposide, lysed and then incubated with alkaline phosphatase after immunoprecipitation. Treatment with alkaline phosphatase abolished the effect on MEF2, indicating that these changes were most likely due to phosphorylation (Figure 3-2). I also treated cerebellar granule cell neurons with two stressors that do not induce DSBs, ultraviolet (UV) light or staurosporine (STS) (a kinase inhibitor). Neither caused an increase ATM-phosphorylated MEF2D protein levels (Figure 3-3).

ATM plays a pivotal role in the immediate response of cells to DSBs (Khanna *et al*., 2001; Shiloh, 2003). To examine whether ATM mediates the phosphorylation of MEF2D after DNA damage, I prepared cerebellar granule cell neurons from ATM wild-type and knockout mice and then treated the cells with etoposide or left them untreated. I found that ATM-phosphorylated MEF2D proteins were detected in *Atm* wild-type cells but not in *Atm*-deficient cells after etoposide treatment (Figure 3-4). Taken together,

these results suggest that DNA damaging agents that induce DSBs trigger the phosphorylation of MEF2, and this phosphorylation is mediated by ATM.

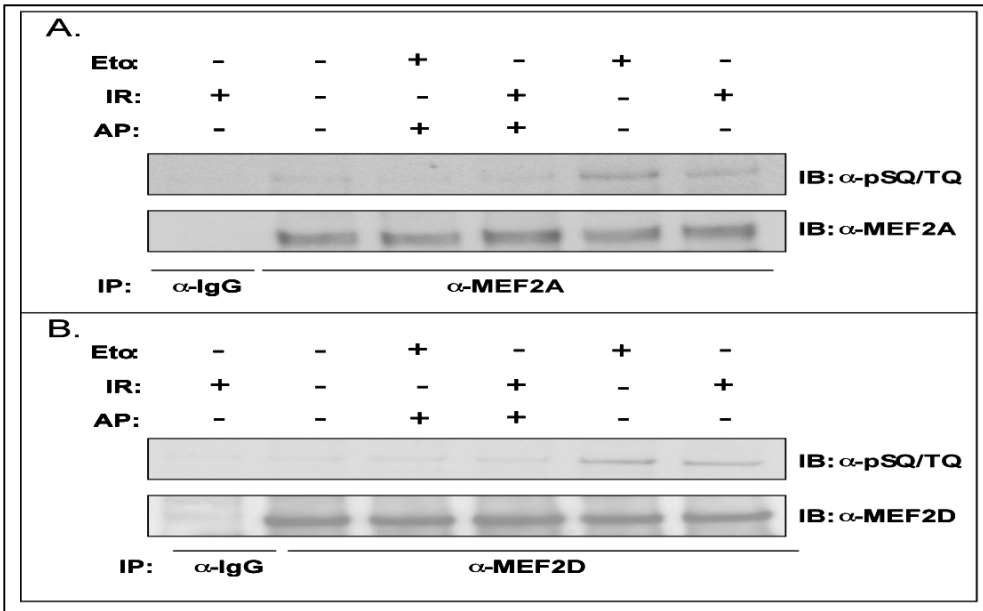

Figure 3-2. DNA damaging agents increase MEF2A and -D phosphorylation levels in cerebellar granule cell neurons.
Cerebellar granule cell neurons were exposed to ionizing radiation (IR, 10 Gy) or etoposide (Eto, 10 μM for 2 h) or left untreated. The lysed cells were immunoprecipitated (IP) using (A) anti-MEF2A or (B) anti-MEF2D antibodies and then separated by SDS-PAGE. Alkaline phosphatase (AP, 10 U at 37 ^{o}C for 1 h) was added after immunoprecipitation to dephosphorylate ATM-phosphorylated-MEF2A and -D proteins in the indicated samples. ATM-phosphorylated MEF2A and -D proteins were detected by immunoblotting (IB) with anti-phospho-SQ/TQ substrate antibody. Anti-IgG antibody was used as a control for immunoprecipitation. Expression of total MEF2A and -D proteins was detected by immunoblotting with anti-MEF2A and anti-MEF2D antibodies respectively.

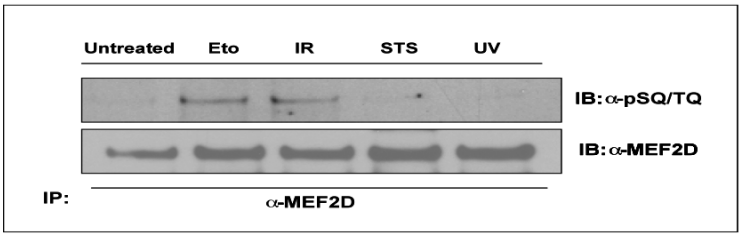

Figure 3-3. DNA damaging agents that induce double-strand breaks trigger MEF2D phosphorylation in cerebellar granule cell neurons.
Cerebellar granule cell neurons were exposed to ionizing radiation (IR, 10 Gy) or etoposide (Eto, 10 μM for 2 h) or staurosporine (STS, 0.1 μM for 2 h) or ultraviolet light (UV, 10 J/m^2) or left untreated. MEF2D proteins were immunoprecipitated (IP) from lysed cells using anti-MEF2D antibody and then separated by SDS-PAGE. ATM-phosphorylated MEF2D proteins were detected by immunoblotting (IB) with anti-phospho-SQ/TQ substrate antibody. Expression of total MEF2D proteins was detected by immunoblotting with anti-MEF2D antibody.

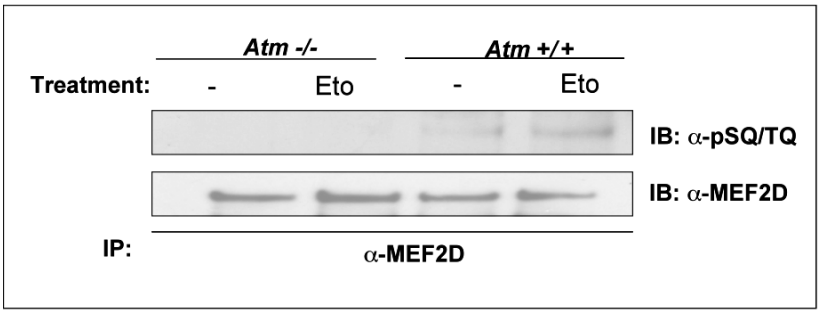

Figure 3-4. MEF2D phosphorylation is detectable in *Atm* wild-type cells but not in *Atm*-deficient cells.
Cerebellar granule cell neurons from *Atm* wild-type mice and knockout mice were exposed to etoposide (Eto, 10 μM for 2 h) or left untreated. MEF2D proteins were immunoprecipitated (IP) from lysed cells using anti-MEF2D antibody and then separated by SDS-PAGE. ATM-phosphorylated MEF2D proteins were detected by immunoblotting (IB) with anti-phospho-SQ/TQ substrate antibody. Expression of total MEF2D proteins was detected by immunoblotting with anti-MEF2D antibody.

3.3. ATM Modulates MEF2 Activity After Exposure of Cells to DNA Damaging Agents

The MEF2 activity and function are governed in part by phosphorylation (McKinsey *et al*., 2002; Flavell and Greenberg, 2008). It is therefore of interest to investigate how the phosphorylation of MEF2 by ATM affects its MEF2 activity. I used GAL4-dependent luciferase reporter gene assays to measure the effect of ATM on MEF2A, -C or -D activity after DNA damage. HEK293T cells were first transfected with two reporter constructs, GAL4-luc and *Renilla*, and an expression vector encoding the DNA binding domain of the transcription factor protein GAL4 fused to the transactivation domain of MEF2A, -C or -D, plus either w.t ATM and/or k.d. ATM. The cotransfected cells were then exposed to IR or left untreated before luciferase activity was measured. After IR, MEF2A and -D activity were significantly activated in cells

overexpressing w.t. ATM (Figure 3-5). The addition of k.d. ATM substantially attenuated the increase in MEF2A and -D activity produced by IR. The k.d. ATM alone prevented the activation of MEF2A and -D activity after IR (Figure 3-5). In contrast, w.t. ATM significantly downregulated MEF2C activity after IR, whereas MEF2C activity was considerably enhanced by the addition of k.d. ATM (Figure 3-5). Since w.t. ATM potentiated MEF2A and -D activity but down-regulated MEF2C activity, these results suggest that ATM may exert a different and opposing effect on different MEF2 family members.

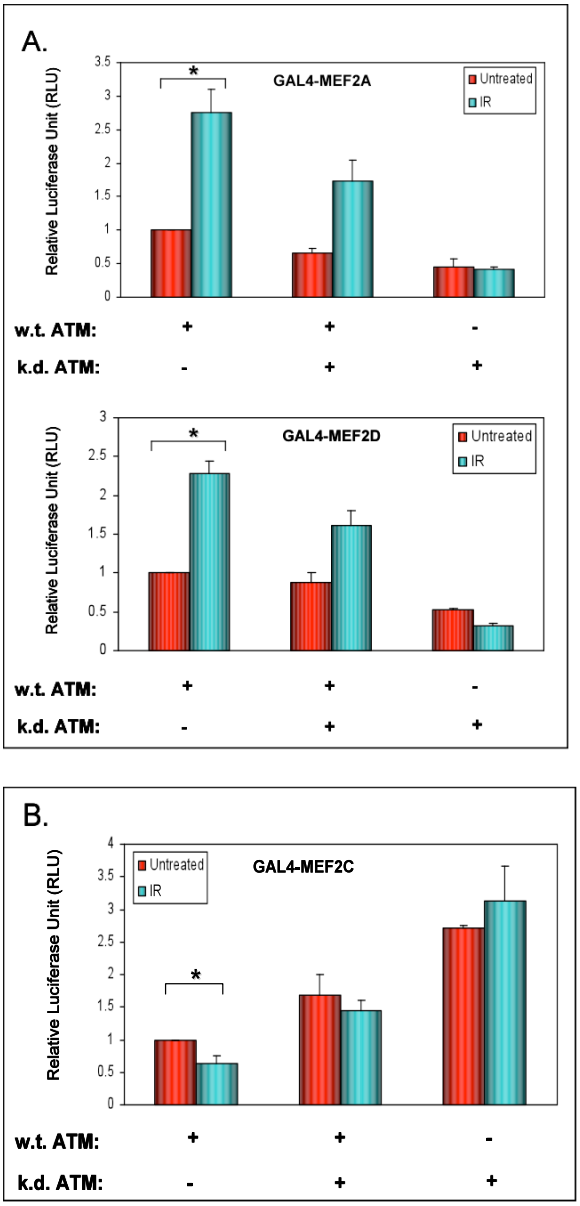

Figure 3-5. ATM regulates MEF2 activity in GAL4-dependent luciferase reporter gene assays.

HEK293T cells were co-transfected with the following expression plasmids: a reporter construct (GAL4-luc), an expression vector encoding the DNA binding domain of GAL4 fused to the transactivation domain of MEF2A or -D (A), or -C (B), and either wild-type (w.t., catalytically active) and/or kinase-dead (k.d., dominant negative) ATM. One day after transfection, cells were exposed to ionizing radiation (IR, 10 Gy) or left untreated before luciferase activity was measured. *Renilla* expression plasmids were used to control for transfection efficiency. The basal luciferase activity of untreated cells that were transfected with w.t. ATM was arbitrarily set equal to one, and all other values were normalized to this reference point. Data are presented as mean ± SD. An asterisk indicates a statistically significant difference between the indicated groups, $P < 0.01$.

MEF2 forms homo- and hetero-dimers (Black and Olson, 1998). Abundance of MEF2 and alterations in the stoichiometry or composition of MEF2 dimers may affect endogenous MEF2 activity. In addition, MEF2A and -D predominate in the cerebellum, which is most affected in A-T. Therefore, I used a second reporter gene system with a MEF2-dependent luciferase reporter gene (Okamoto *et al*., 2002) to determine how ATM regulates endogenous MEF2 activity in cerebellar granule cell neurons. The MEF2-dependent luciferase reporter gene was constructed by insertion of two tandem copies of the MEF2 site from the brain creatine kinase enhancer into the upstream of the basal promoter of SV40 and was able to measure endogenous MEF2 activity (Okamoto *et al*., 2002). Two experimental approaches were used to examine the regulation of endogenous MEF2 activity by ATM: i) knocking down ATM expression using RNA interference (RNAi), and ii) pharmacologically inhibiting ATM kinase activity using a small molecule inhibitor of ATM, KU55933.

I first used a U6 promoter-driven short-hairpin RNA (shRNA) to knockdown ATM expression and examined the subsequent biological effects on endogenous MEF2 activity in cerebellar granule cell neurons using a MEF2-dependent luciferase reporter gene assay. The shRNA expression vector contains an internal green fluorescent marker for monitoring delivery efficiency of shRNA-expressing constructs. Cerebellar granule cell neurons were cotransfected with a reporter construct (MEF2-luc), plus one of two shRNAs targeting ATM (shRNA-1 ATM or shRNA-2 ATM) or a scrambled shRNA as a control. Exposure to IR or etoposide activated endogenous MEF2 activity. Unlike non-target shRNA, scrambled shRNA or shRNA-2 ATM, shRNA-1 ATM inhibited MEF2 activation under these conditions (Figure 3-6A). Immunostaining with an anti-ATM

antibody confirmed that shRNA-1 ATM efficiently inhibited ATM expression in cerebellar granule cell neurons (Figure 3-6B). To further confirm that ATM activated endogenous MEF2 activity after DNA damage, I used a cell-permeable small molecule inhibitor of ATM, KU-55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one) to inhibit ATM kinase activity (Hickson *et al*., 2004). KU55933 abrogated IR-induced activation of endogenous MEF2 activity (Figure 3-7A). Immunostaining with the anti-phospho-SQ/TQ substrate antibody confirmed that KU55933 abated ATM kinase activity in cerebellar granule cell neurons (Figure 3-7B).

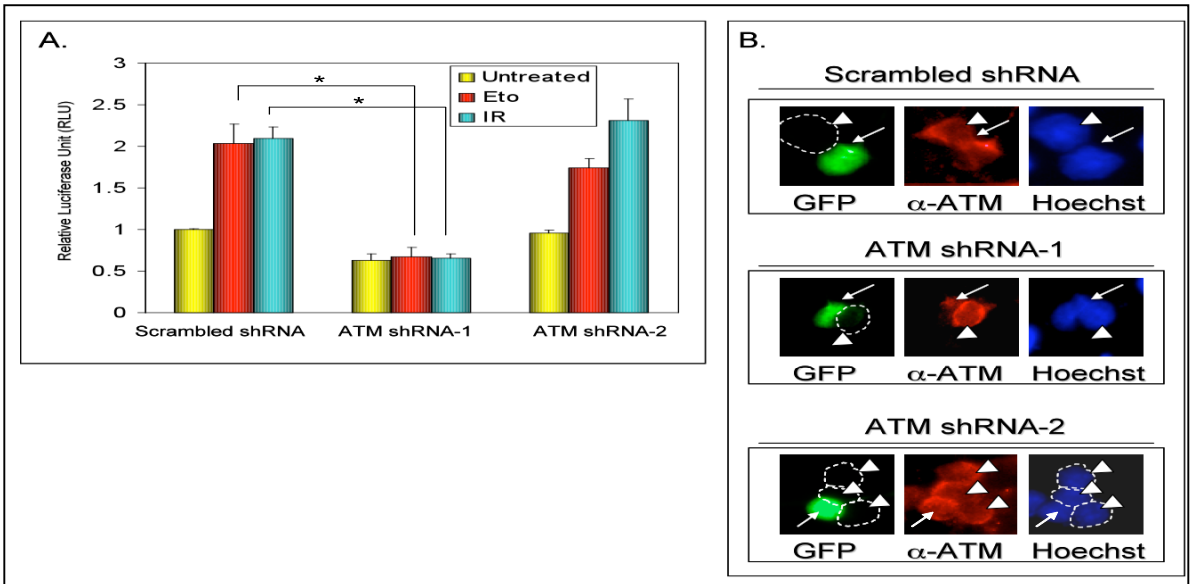

Figure 3-6. An shRNA targeting ATM reduces the potentiation of MEF2 activity after exposure to DNA damaging agents.
(A) Cerebellar granule cell neurons were co-transfected with plasmids expressing a reporter construct (MEF2-luc), plus one of two shRNAs targeting ATM (shRNA-1 ATM or shRNA-2 ATM) or a scrambled shRNA. Two days after transfection, cells were exposed to ionizing radiation (IR, 10 Gy), etoposide (Eto, 10 μM for 2 h) or left untreated prior to measuring luciferase activity. *Renilla* expression plasmids were used to control for transfection efficiency. The basal luciferase activity of untreated cells that were transfected with scrambled shRNA was arbitrarily set equal to one, and all other values were normalized to this reference point. Data are presented as mean ± SD. An asterisk indicates a statistically significant difference between the indicated groups, $P < 0.01$. (B) Cerebellar granule cell neurons were transfected with an shRNA targeting ATM (either shRNA-1 ATM or shRNA-2 ATM) or a scrambled shRNA. Two days after transfection, cells were immunostained with anti-ATM antibody to detect ATM expression, and Hoechst dye 33242 was used to visualize cell nuclei. Arrows indicate cells transfected with shRNA, and triangles and white outlines represent untransfected cells.

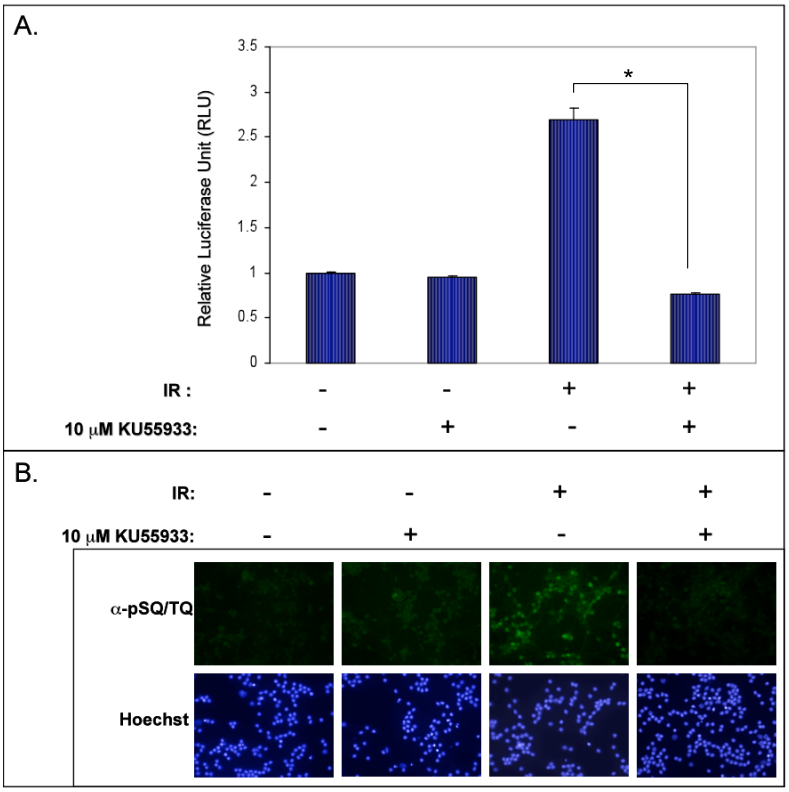

Figure 3-7. A small molecule inhibitor of ATM, KU55933 abates the augmentation of MEF2 activity after exposure to ionizing radiation.
(A) Cerebellar granule cell neurons were exposed to ionizing radiation (IR, 10 Gy) in the presence or absence of KU55933 (10 μM), following which luciferase activity was measured. *Renilla* expression plasmids were used to control for transfection efficiency. The basal luciferase activity of untreated cells was arbitrarily set equal to one, and all other values were normalized to this reference point. Data are presented as mean ± SD. An asterisk indicates a statistically significant difference between the indicated groups, $P < 0.01$. (B) Cerebellar granule cell neurons were immunostained with anti-phospho-SQ/TQ substrate antibody to detect the activation of ATM kinase activity after exposure to IR in the presence or absence of KU55933. Hoechst dye 33242 was used to visualize cell nuclei.

3.4. Identification of ATM-phosphorylatable Sites in MEF2D

Of the four members of the MEF2 family, MEF2A and -D are expressed predominately in the cerebellum, which is most affected brain area in A-T. Previous studies have shown that MEF2A plays a critical role in promoting neuronal survival

(Gaudilliere *et al*., 2002; Flavell and Greenberg, 2008); however, a specific role for MEF2D in cerebellar granule cell neuronal survival is as yet unclear. Elucidating the function of MEF2D in neuronal survival in the face of DNA damage is the focus of the remaining sessions of this thesis dissertation.

MEF2D contains four putative ATM consensus phosphorylation motifs (SQ/TQ) (Figure 1-2). To determine which of the four serine/threonine residues in MEF2D are phosphorylated by ATM, I used the QuikChange multi site-directed mutagenesis kit (Stratagene) to mutate them into alanines (A), either singly or in combination (i.e., changing the SQ/TQ motifs into AQ) (Figure 3-8). Mutations were confirmed by DNA sequencing. Each of the MEF2D mutants and w.t. MEF2D were expressed as V5-tagged fusion proteins. HEK293T cells that had been transfected with expression plasmids encoding either w.t. MEF2D or each of the MEF2D mutants (Figure 3-8) were exposed to IR, and cell extracts were immunoprecipitated with an anti-V5 antibody. ATM-phosphorylated MEF2D proteins were detected by immunoblotting with the anti-phospho-SQ/TQ substrate antibody.

I found that the ATM-phosphorylated MEF2D protein level was completely abolished in the MEF2D mutant that lacked all four putative ATM-phosphorylatable sites, which represents a nonphosphorylatable MEF2D mutant (T259A/S275A/S294A/S314A) (Figure 3-9). In contrast, the ATM-phosphorylated MEF2D protein level was similar to that w.t MEF2D in each of the four MEF2D mutants that contained three putative ATM-phosphorylatable sites and a single alanine substitution (T259A, S275A, S294A or S314A) and in each of the four MEF2D mutants that possessed only one putative ATM phosphorylatable sites and three alanine substitutions (T259/S275A/S294A/S314A,

T259A/S275/S294A/S314A, T259A/S275A/S294/S314A or T259A/S275A/S294A/S314) (Figure 3-9). These results suggest that ATM may phosphorylate MEF2D at four consensus ATM-phosphorylatable sites: Thr^{259}, Ser^{275}, Ser^{294} or Ser^{314}.

The four consensus ATM-phosphorylatable sites in MEF2D are all located in the transactivation domain, which is crucial for MEF2 activity (Figure 1-2). It is therefore of interest to examine which ATM-phosphorylatable sites in MEF2D are essential for MEF2 activity after exposure to DNA damage. HEK293T cells were first transfected with two reporter constructs, MEF2-luc and *Renilla*, and an expression vector encoding w.t. MEF2D, the nonphosphorylatable MEF2D mutant, or each of the four MEF2D mutants that contained three ATM-phosphorylatable sites and a single alanine substitution. The transfected cells were then exposed to etoposide or left untreated before luciferase activity was measured.

After etoposide exposure, MEF2 activity was substantially increased in cells expressing w.t. MEF2D, but the enhancement of MEF2 activity was not observed in cells expressing the nonphosphorylatable MEF2D mutant (Figure 3-10). These findings suggest that the nonphosphorylatable MEF2D mutant cannot be activated by DNA damage and therefore remains inactive. Expression of each of the four MEF2D mutants that contained three ATM-phosphorylatable sites and a single alanine substitution also induced MEF2 activity after etoposide exposure, but the increase was much less than with w.t. MEF2D (Figure 3-10). These results suggest that each of the four ATM-phosphorylatable sites in MEF2D, i.e., Thr^{259}, Ser^{275}, Ser^{294} and Ser^{314}, contributes to full activation after DNA damage.

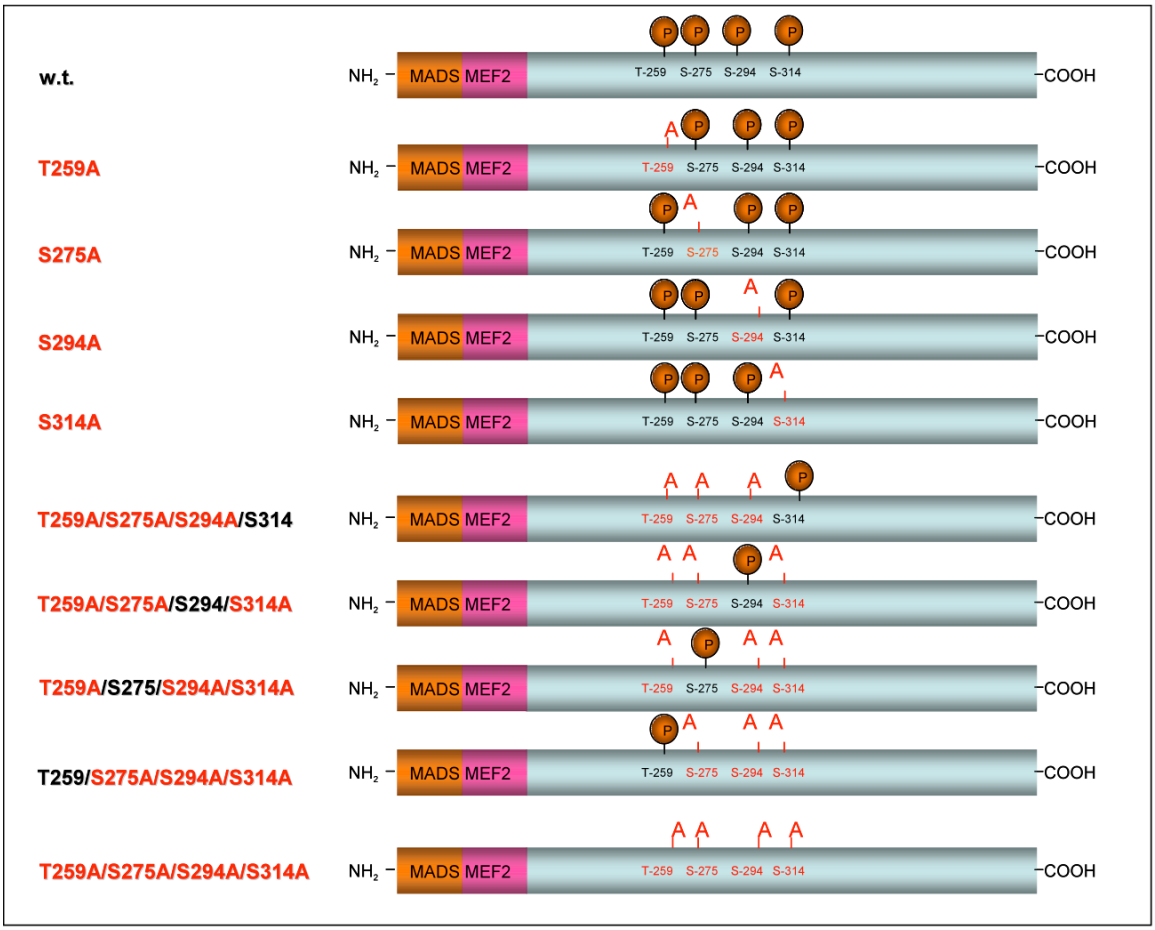

Figure 3-8. Alanine substitutions at ATM-phosphorylatable sites in MEF2D.
Serine/threonine residues of potential ATM-phosphorylatable sites (SQ/TQ) in MEF2D were mutated into alanine. Left: The four potential ATM-phosphorylatable sites in MEF2D are listed; alanine substitutions are indicated with an "A" after the residue number and are highlighted in red color. Right: Potential ATM-phosphorylatable sites in MEF2D are marked with a "P". Alanine substitutions are indicated by replacement of the "P" with an "A".

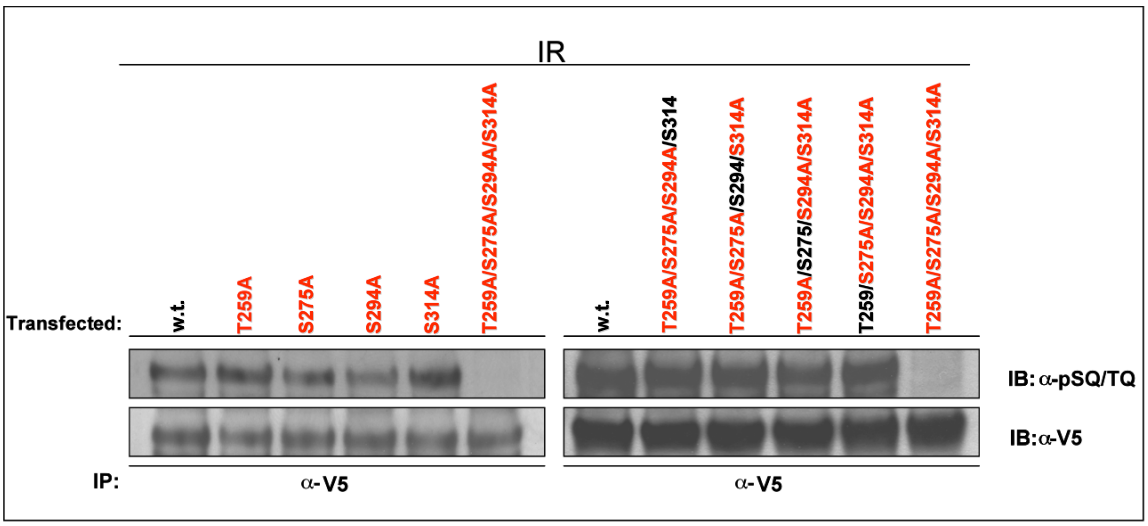

Figure 3-9. Identification of ATM-phosphorylatable sites in MEF2D.
HEK293T cells were transfected with plasmids expressing wild-type (w.t.) MEF2D or MEF2D mutants containing alanine substitutions at phosphorylatable sites, each tagged with V5. The transfected cells were then exposed to ionizing radiation (IR, 10 Gy). Cell extracts from the transfected cells were immunoprecipitated (IP) with anti-V5 antibody. ATM-phosphorylated MEF2D proteins were detected by immunoblotting (IB) with anti-phospho-SQ/TQ substrate antibody. Expression of total MEF2D proteins was detected by immunoblotting with anti-V5 antibody.

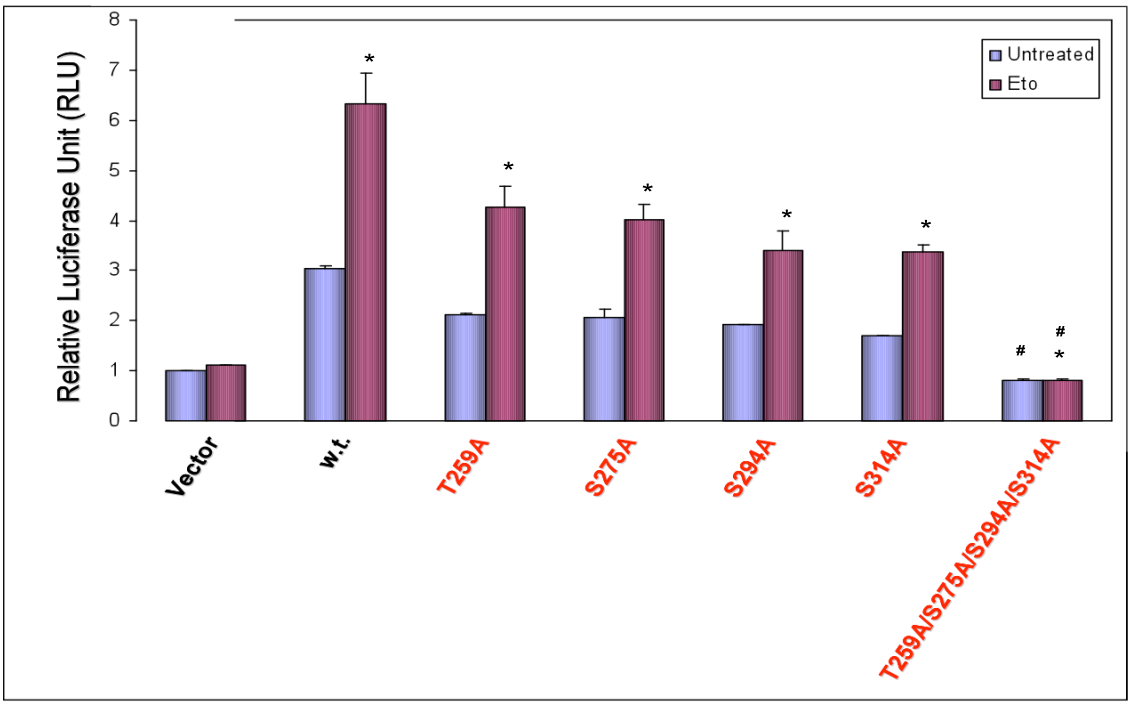

Figure 3-10. Each of the four ATM-phosphorylatable sites in MEF2D contributes to full activation after DNA damage.
HEK293T cells were co-transfected with the following expression plasmids: two reporter constructs, MEF2-luc and *Renilla*, plus wild-type (w.t.) MEF2D, a nonphosphorylatable MEF2D mutant (T259A/S275A/S294A/S314A), a MEF2D mutant containing three putative ATM-phosphorylatable sites and a single alanine substitution (T259A, S275A, S294A or S314A) or pcDNA3 vector control. One day after transfection, cells were exposed to etoposide or left untreated prior to measuring luciferase activity. *Renilla* expression plasmids were used to control for transfection efficiency. Transfection of pcDNA3 was used as a vector control. Basal luciferase activity of untreated vector control was arbitrarily set equal to one, and all other values were normalized to this reference point. Data are presented as mean ± SD. An asterisk indicates a statistically significant difference between the indicated groups, $P < 0.01$. The number sign indicates that there was no statistically significant difference between the indicated groups.

3.5. Investigation of the Physiological Effect of ATM-dependent Phosphorylation/Activation of MEF2D for Neuronal Survival in Response to DNA Damage

To elucidate the potential role of ATM-mediated phosphorylation/activation of MEF2D for neuronal survival, I first investigated whether MEF2 activity is affected by the phospho-mimicking mutation in MEF2D. Two phospho-mimetic MEF2D mutants

were constructed by converting all four ATM-phosphorylatable sites in MEF2D into either aspartic or glutamic acid residues (i.e., by changing the SQ/TQ motifs into either DQ or EQ). The functional consequence of this phospho-mimetic mutation on MEF2 activity was then assessed using the MEF2-dependent luciferase reporter gene assays.

HEK293T cells were cotransfected with two reporter constructs, MEF2-luc and *Renilla*, plus either w.t. MEF2D, the nonphosphorylatable MEF2D mutant, one of two phospho-mimetic MEF2D mutants (T259D/S275D/S294D/S314D or T259E/S275E/S294E/S314E), a constitutively active MEF2 (MEF2CA) or a vector control. The transfected cells were then exposed to IR or left untreated prior to measuring luciferase activity. Cells expressing phospho-mimetic MEF2D mutant with aspartic acid substitutions (T259D/S275D/S294D/S314D) showed an elevated MEF2 activity without IR treatment, but cells expressing phospho-mimetic MEF2D mutant with glutamic acid substitutions (T259E/S275E/S294E/S314E) did not (Figure 3-11A). As expected, MEF2 was inactive in cells expressing the nonphosphorylatable MEF2D mutant, whereas cells expressing w.t. MEF2D manifested an increased MEF2 activity in response to IR (Figure 3-11A). MEF2CA, which encodes the DNA-binding and dimerization domain of MEF2 fused to a fully active VP16 transactivation domain, was used as a positive control in these experiments and displayed very high MEF2 activity compared to vector control (Figure 3-11B). Taken together, these results suggest that MEF2D mutant with aspartic acid substitutions (T259D/S275D/S294D/S314D) can mimic IR-induced MEF2 activation.

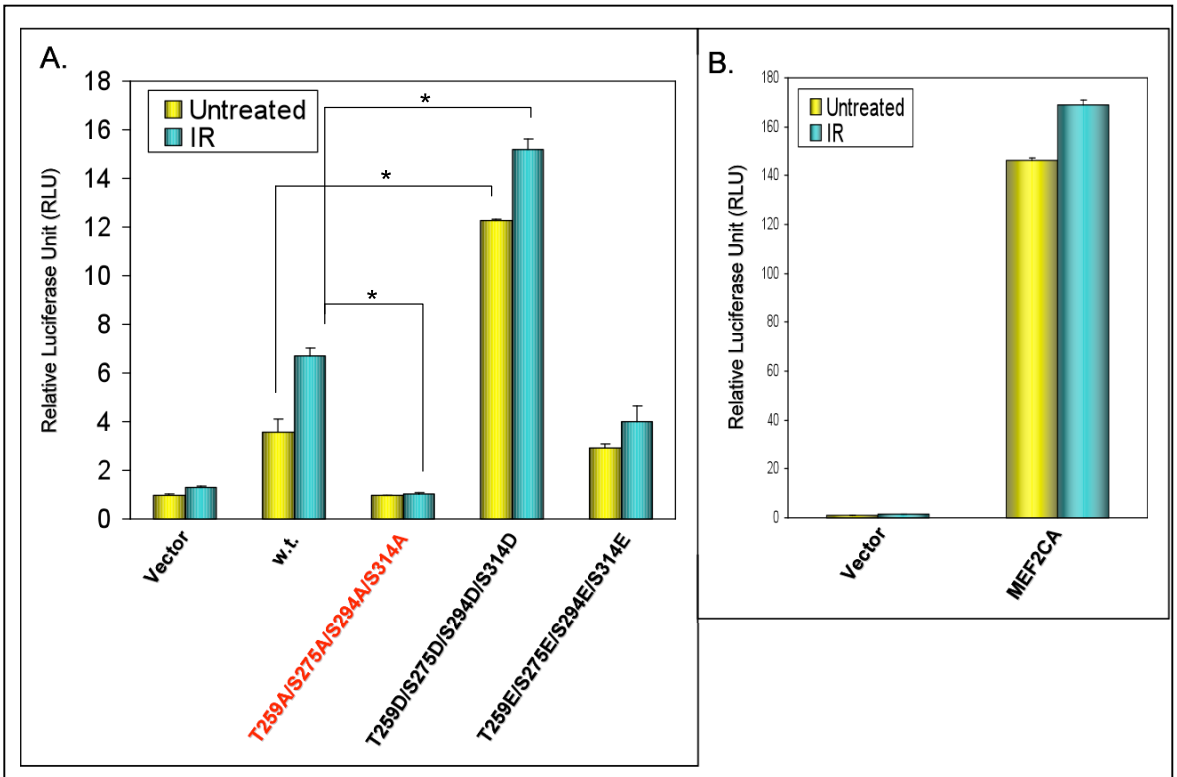

Figure 3-11. Effect of nonphosphorylatable and phospho-mimetic mutations of MEF2D on MEF2 activity.
(A) HEK293T cells were co-transfected with the following expression plasmids: two reporter constructs, MEF2-luc and *Renilla*, plus either wild-type (w.t.) MEF2D, a nonphosphorylatable MEF2D mutant (T259A/S275A/S294A/S314A), a phospho-mimetic MEF2D mutant (either T259D/S275D/S294D/S314D or T259E/S275E/S294E/S314E) or pcDNA3 vector control. One day after transfection, transfected cells were exposed to ionizing radiation (IR, 10 Gy) or left untreated prior to measuring luciferase activity. *Renilla* expression plasmids were used to control for transfection efficiency. Transfection of pcDNA3 was used as a vector control. Basal luciferase activity of untreated vector control was arbitrarily set equal to one, and all other values were normalized to this reference point. (B) Constitutively active MEF2 (MEF2CA) was used as a positive control. Data are presented as mean ± SD. An asterisk indicates a statistically significant difference between the indicated groups, $P < 0.01$.

Because activation of MEF2 is critical for neuronal survival (Okamoto *et al.*, 2000; Gaudilliere *et al.*, 2002; Shalizi *et al.*, 2003; Flavell and Greenberg, 2008), I investigated whether cerebellar granule cell neuronal survival after etoposide exposure is affected by knockdown of endogenous MEF2D expression with an shRNA targeting MEF2D (shRNA-MEF2D) and by substitution of endogenous MEF2D expression with

one of three shRNA-resistant forms of MEF2D (w.t. MEF2D, a nonphosphorylatable MEF2D mutant, or a phospho-mimetic MEF2D mutant (T259D/S275D/S294D/S314D)). A strategy that combines the use of RNAi and overexpression of shRNA-resistant MEF2D mutants can thus define the physiological effect of ATM-dependent phosphorylation/activation of MEF2D on neuronal survival after DNA damage.

I first constructed four different shRNA-MEF2D and examined their ability to knock down transient overexpression of MEF2D in mammalian cells as well as endogenous MEF2D expression in cerebellar granule cell neurons. HEK293T cells were transfected with w.t. MEF2D, plus one of four targeted shRNAs, (shRNA-MEF2D-172, -176, -187 or -436) or scrambled shRNA as a control. Western blot analyses showed that shRNA-MEF2D-176, -187 and -436 inhibited MEF2D expression in mammalian cells, but shRNA-MEF2D-172 and shRNA-scrambled did not (Figure 3-12A). Immunostaining with an anti-MEF2D antibody confirmed that shRNA-MEF2D-176, -187 and -436 efficiently inhibited endogenous MEF2D expression in cerebellar granule cell neurons, but shRNA-MEF2D-172 and shRNA-scrambled did not (Figure 3-12B).

After confirming that RNAi of MEF2D can knock down exogenous and endogenous MEF2D expression, I constructed an expression plasmid encoding either one of three shRNA-resistant forms of MEF2Ds, w.t. MEF2D, a nonophosphorylatable MEF2D mutant, or a phospho-mimetic MEF2D mutant. Silent mutations were introduced at the shRNA-MEF2D target site in MEF2D (the sequence recognized by shRNA-MEF2D-436). Five such mutations were confirmed by DNA sequencing. I then examined whether these silent mutations could provide resistance against shRNA-MEF2D (Figure 3-13). Two of the shRNA-resistant forms of w.t. MEF2D (designated

MEF2D-4 and -5), in addition to nonphosphorylatable MEF2D-4 and -5 mutants and phospho-mimetic MEF2D-4 and -5 mutants displayed resistance to this shRNA-MEF2D. In contrast, mutants 1-3 of the shRNA-resistant form of w.t., nonphosphorylatable or phospho-mimetic MEF2D were not resistant to this shRNA-MEF2D (Figure 13-14A, B and C).

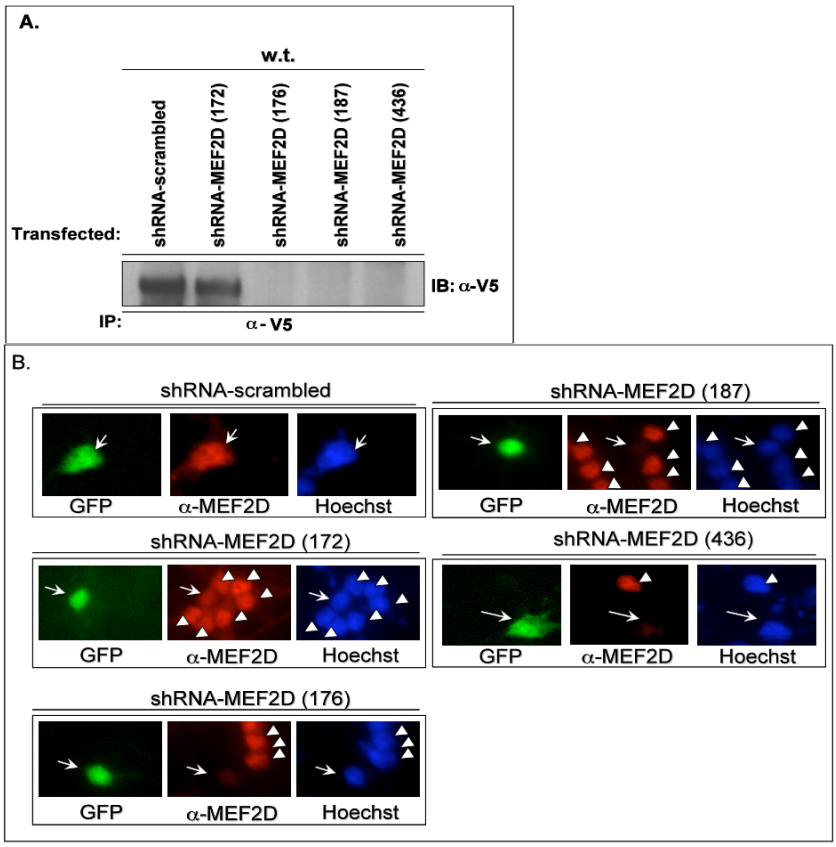

Figure 3-12. shRNAs targeting MEF2D inhibit MEF2D expression in mammalian cell lines and cerebellar granule cell neurons.
(A) HEK293T cells were transfected with plasmids expressing wild-type (w.t.) MEF2D, plus either a scrambled shRNA or an shRNA targeting MEF2D (172, 176, 187, or 436). Two days after transfection, cell extracts from the transfected cells were immunoprecipitated (IP) with anti-V5 antibody. MEF2D expression was then detected by immunoblotting (IB) with anti-V5 antibody. (B) Cerebellar granule cell neurons were transfected with an shRNA targeting MEF2D (172, 176, 187, or 436) or a scrambled shRNA. Two days after transfection, cells were immunostained with anti-MEF2D antibody to detect MEF2D expression, and Hoechst dye 33242 to visualize cell nuclei. Arrows indicate cells transfected with shRNA, and triangles represent untransfected cells.

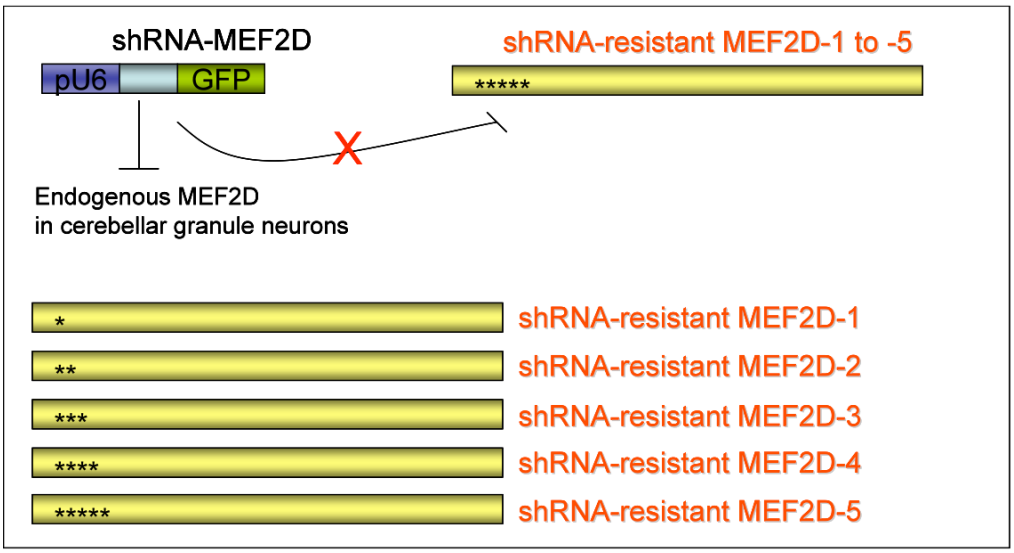

Figure 3-13. Design of shRNA-resistant forms of MEF2D cDNA.
Silent mutations were introduced at the shRNA-MEF2D target sites of the MEF2D expression plasmid. Each silent mutation in the MEF2D expression plasmid is marked with an asterisk.

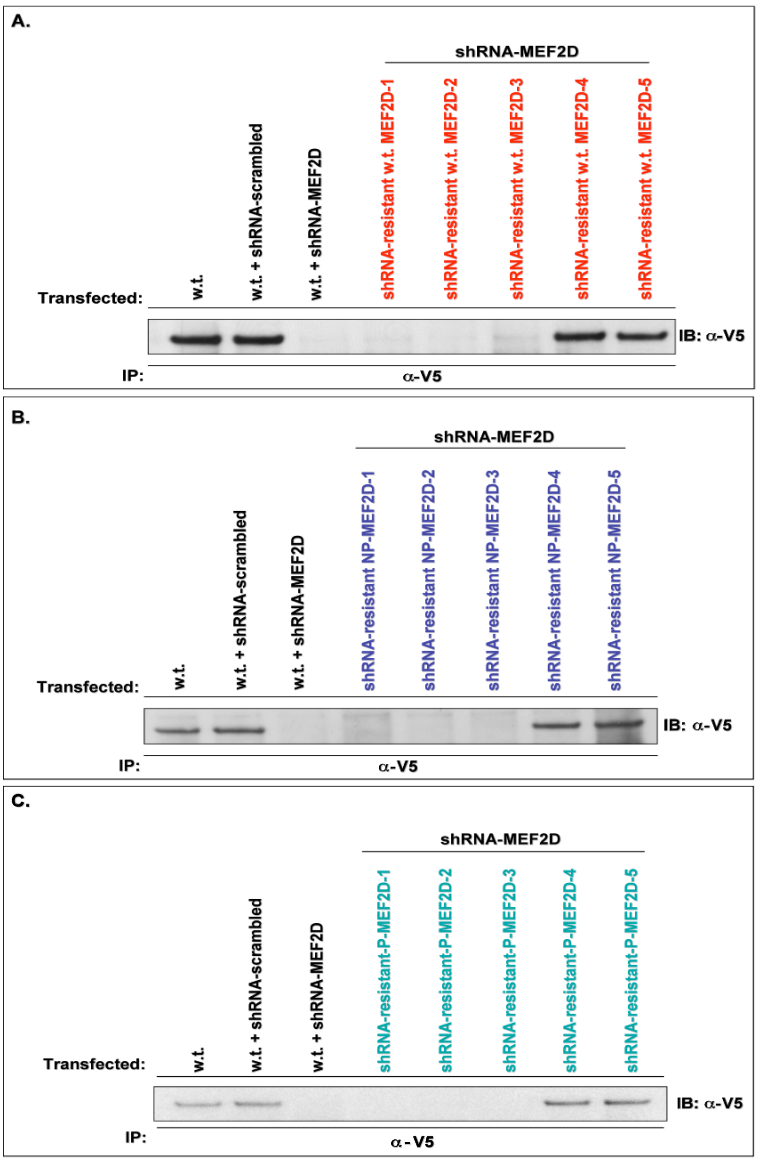

Figure 3-14. shRNA-resistant forms of MEF2D cDNA manifest resistance to shRNA targeting MEF2D.

HEK293T cells were transfected with plasmids expressing an shRNA targeting ME2D, plus (A) shRNA-resistant form of wild-type (w.t.) MEF2D-1, -2, -3, -4 or -5, (B) shRNA-resistant form of nonphosphorylatable (NP) MEF2D-1, -2, -3, -4 or -5, (C) shRNA-resistant form of phospho-mimetic (P) MEF2D-1, -2, -3, -4 or -5. Additionally, cells were transfected with w.t. MEF2D plus scrambled shRNA or shRNA targeting ME2D as a control. Two days after transfection, cell extracts from the transfected cells were immunoprecipitated (IP) with anti-V5 antibody. MEF2D expression was then detected by immunoblotting (IB) with anti-V5 antibody.

With these tools, I was able to substantiate the significance of ATM-mediated MEF2D phosphorylation and activation on neuronal survival in the face of DNA damage. RNAi of MEF2D was first used to knock down endogenous MEF2D expression in cerebellar granule cell neurons. Subsequently, I substituted for the depleted w.t. MEF2D expression with an shRNA-resistant form of either w.t. MEF2D, nonphosphorylatable MEF2D mutant, phospho-mimetic MEF2D mutant or an shRNA-scrambled served as a control. I then quantified apoptotic cell death of transfected cerebellar granule cell neurons exposed to etoposide.

Cerebellar granule cell neurons expressing the shRNA-MEF2D were more susceptible to etoposide-induced cell death than the neurons expressing control shRNA-scrambled (Figure 3-15). Interestingly, substitution of endogenous MEF2D expression with an shRNA-resistant form of phospho-mimetic MEF2D mutant protected from cell death after etoposide exposure to an even greater degree than shRNA-resistant form of w.t. MEF2D. In contrast, substitution with an shRNA-resistant form of nonphosphorylatable MEF2D mutant was not neuroprotective (Figure 3-15). A dominant-negative MEF2 (MEF2DN), which was previously shown to inhibit MEF2 activity and enhance cell death after NMDA exposure (Okamoto *et al*., 2000), also increased cell death after etoposide treatment, whereas MEF2CA prevented cell death (Figure 3-15). Note that this MEF2DN exerts its dominant interference by binding to the MEF2 DNA binding site without producing activation because it lacks the transactivation domain (Molkentin *et al*., 1996). Taken together, these results are consistent with the notion that knockdown of endogenous MEF2D expression increases cellular sensitivity to etoposide-induced neuronal cell death in the cerebellum. Neuronal rescue by

overexpression of shRNA-resistant form of phospho-mimetic MEF2D mutant, but not shRNA-resistant form of nonphosphorylatable MEF2D mutant, strongly suggests that ATM-induced phosphorylation/activation of MEF2 is essential for neuronal survival in the face of DNA damage.

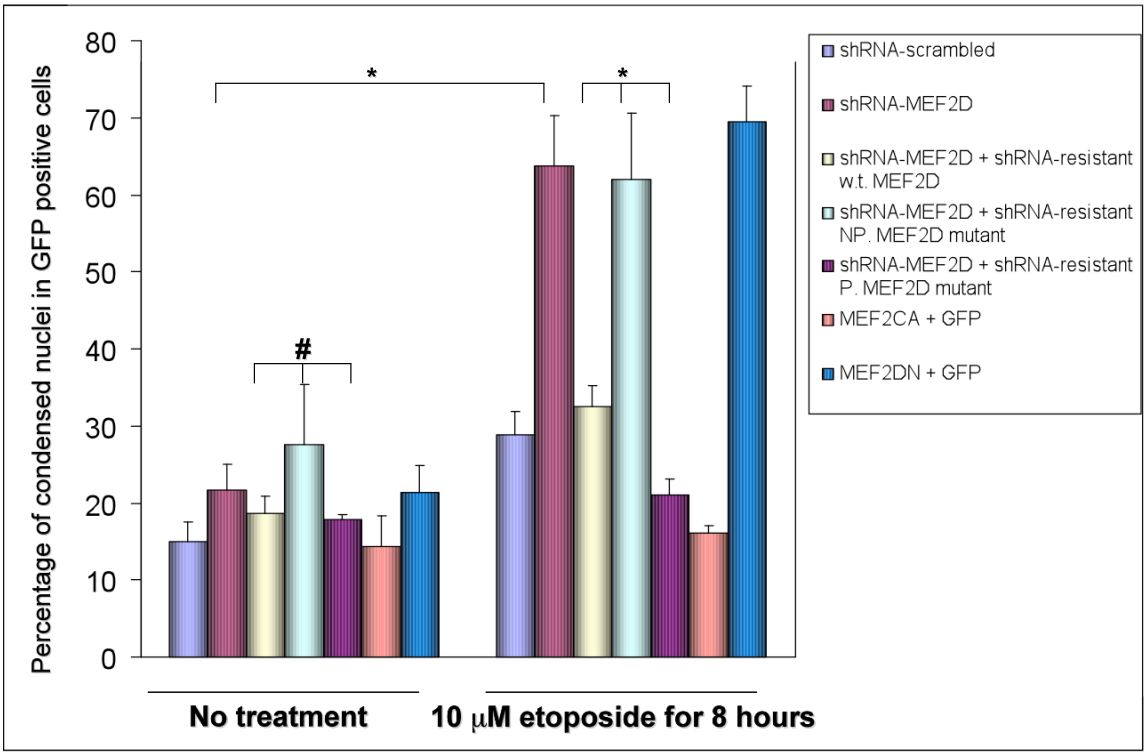

Figure 3-15. Cerebellar granule cell neurons expressing an shRNA targeting MEF2D are more susceptible to etoposide-induced cell death.
Cerebellar granule cell neurons were transfected with the following expression plasmids: scrambled shRNA or shRNA targeting MEF2D (shRNA-MEF2D), plus either shRNA-resistant form of wild-type (w.t.) MEF2D, nonphosphorylatable (NP) MEF2D mutant or phospho-mimetic (P) MEF2D mutant. Additionally, cerebellar granule cell neurons were transfected with GFP-tagged vector, plus either constitutively active MEF2 (MEF2CA) or dominant-negative MEF2 (MEF2DN). Three days after transfection, granule cell neurons were exposed to etoposide of left untreated. The number of transfected granule cell neurons labeled with GFP was counted for each conditions. Hoechst dye 33342 was used to identify condensed nuclei indicative of apoptotic cell death. Data are presented as mean ± SD. Asterisks indicate a statistically significant difference between the indicated groups, $P < 0.05$. The number sign indicates that there was no statistically significant difference between the indicated groups.

3.6. Co-immunoprecipitation of ATM and MEF2D in Mammalian Cells

The ATM-interaction motif, consisting of EE/DD/RY doublets, recruits activated ATM and its associated proteins to DNA damage sites and promotes the phosphorylation by ATM (Falck *et al.*, 2005). MEF2D contains an ATM-interaction motif (Figure 3-16A). To test whether ATM forms a complex with MEF2D, HEK293T cells were transiently

transfected with an expression vector encoding V5 tagged w.t. MEF2D. The transfected cells were exposed to IR or left untreated. Cell extracts from transfected cells were then immunoprecipitated with either control antibody (anti-IgG), an anti-ATM or an anti-V5 antibody and immunoblotted with anti-ATM antibody to detect ATM expression. The anti-V5 antibody co-immunoprecipitated MEF2D and endogenous ATM in cells exposed to IR, but not in untreated cells (Figure 3-16B). These results suggest that ATM and MEF2D form a complex in response to DNA damage.

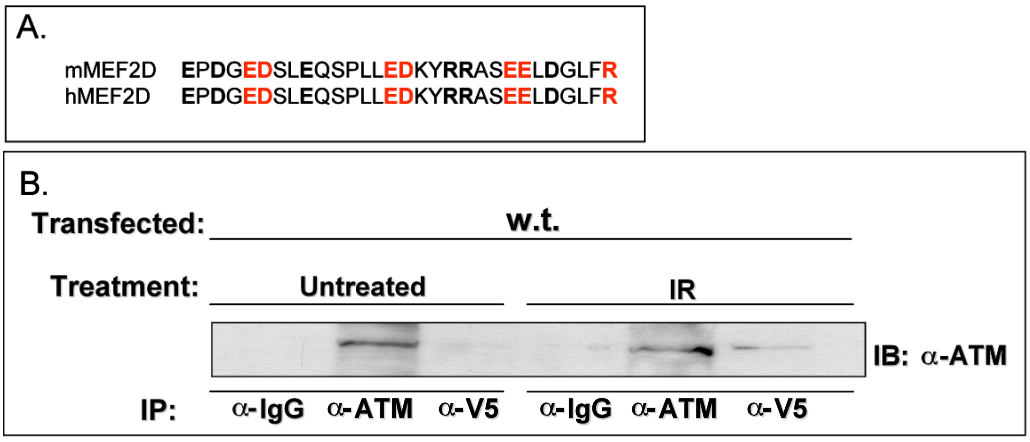

Figure 3-16. ATM and MEF2D form a complex in response to DNA damage.
(A) Putative ATM-interaction motif in MEF2D. EE/DD/RY doublets are highlighted in red. mMEF2D, mouse MEF2D; hMEF2D, human MEF2D. (B) HEK293T cells were transiently transfected with an expression vector encoding V5-tagged w.t. MEF2D. Two days after transfection, cells were exposed to ionizing radiation (IR, 10 Gy) or left untreated. Cell extracts from the transfected cells were immunoprecipitated (IP) with control anti-IgG, anti-ATM, or anti-V5 antibody. Immunoblots were probed with anti-ATM antibody to detect ATM expression.

Chapter 4: Discussion and Future Directions

In the present study, I demonstrated that double-strand breaks (DSBs) in DNA caused by DNA damaging agents, including ionizing radiation (IR) and the topoisomerase II inhibitor etoposide, induce MEF2D phosphorylation *in vitro* and in cerebellar granule cell neurons. ATM phosphorylates MEF2D, thereby stimulating MEF2 activity. ATM and MEF2D form a complex after DNA damage. Most importantly, I found that knockdown of endogenous MEF2D expression increases cellular sensitivity to etoposide-induced neuronal cell death. The cells can be rescued, however, by substitution of endogenous MEF2D with shRNA-resistant phospho-mimetic MEF2D mutant, but not with shRNA-resistant nonphosphorylatable MEF2D mutant. My studies indicate for the first time that MEF2D acts as an ATM effector to promote neuronal survival in response to DNA damage.

ATM preferentially phosphorylates its substrates on serine or threonine residues that precede glutamine residues, so-called SQ/TQ motifs. Many ATM substrates contain multiple SQ/TQ motifs, and multisite phosphorylation of SQ/TQ motifs is required for cell cycle control, DNA repair, apoptosis and transcription regulation (Traven and Heierhorst, 2005; Shiloh, 2006). Analysis by site-directed mutagenesis showed that MEF2D phosphorylation occurs at four consensus ATM-phosphorylatable sites: Thr^{259}, Ser^{275}, Ser^{294} or Ser^{314}. Mutations at any of the four ATM-phosphorylatable sites in MEF2D were sufficient to prevent full activation of MEF2 in response to DNA damage (Figure 3-9 and 3-10). These results suggest that each of the four ATM-phosphorylatable

sites in MEF2D contributes to regulate MEF2 activity in response to DNA damage.

MEF2 activity is tightly regulated by phosphorylation (Flavell and Greenberg, 2008). It is intriguing to note that all four ATM-phosphorylatable sites in MEF2D are located in the transactivation domain, and no known downstream effectors of ATM provide cross-talk with MEF2. Hence, these sites may represent previously unknown phosphorylation elements for the regulation of MEF2 activity by ATM in response to DNA damage.

ATM and Rad3-related kinase (ATR) also has a preference to phosphorylate SQ/TQ motifs in its substrates (Kim *et.al*., 1999; Traven and Heierhorst, 2005; Shiloh, 2006). ATR is mainly activated by ultraviolet light (UV) and stalled replication forks, whereas ATM is specifically activated by DSBs (Shiloh, 2006). In figure 3-3, DNA damaging agents, IR and etoposide, that can induce DSBs, triggered MEF2D phosphorylation, but ultraviolet light did not. In addition, MEF2D phosphorylation levels were only increased in *Atm* wild-type cells but not in *Atm*-deficient cells after etoposide treatment (Figure 3-4). These results suggest that ATM is required to induce MEF2D phosphorylation in response to DSBs in DNA.

DNA damage has been linked with many neurodegenerative diseases (Abner and McKinnon, 2004), and accumulating evidence suggests that ATM plays an important role in promoting survival in response to DNA damage (Kamer *et al*., 2005; Zinkel *et al*., 2005; Wu *et al*., 2006). ATM is essential for repair of DNA damage and maintaining the balance between cell survival and apoptosis (Lee and McKinnon, 2007). In the presence of repairable and minimal DNA damage, cells activate ATM-dependent DNA damage response pathways to promote cell survival, initiate DNA damage repair and cell cycle

checkpoints. Previous studies suggested that both ATM-NF-kappa B and ATM-BID signaling pathways are important for promoting survival after DNA damage (Kamer *et al*., 2005; Zinkel *et al*., 2005; Wu *et al*., 2006). Defects in the DNA damage response and survival signaling in neurons may contribute to the neurodegenerative pathology of A-T. Numerous substrates for ATM have been identified, and most are related to the regulation of cell cycle checkpoints and development of cancer; however, the targets of ATM in the CNS are as yet unclear. My new results suggest that MEF2D is a downstream substrate of ATM and may act as a DNA damage responsive transcription factor to protect cerebellar granule cell neurons from etoposide-induced cell death.

MEF2A and -D are the predominant form of MEF2 expressed in granule cell neurons in the cerebellum. The RNAi-mediated knockdown experiments in cerebellar granule cell neurons reported here indicate that endogenous MEF2D function is necessary for neuronal survival after etoposide treatment (Figure 3-15). Despite the abundant expression of MEF2A in cerebellar granule cell neurons, these data suggest that MEF2A cannot compensate for MEF2D in promoting survival of these neurons after DNA damage. Replacement of endogenous MEF2D with shRNA-resistant phospho-mimetic MEF2D mutant protected neurons from cell death after DNA damage, but shRNA-resistant nonphosphorylatable MEF2D mutant did not (Figure 3-15). These results are consistent with the notion that ATM-mediated phosphorylation/activation of MEF2D may induce pro-survival activity in cells after DNA damage in order to survive the insult and maintain the homeostasis of the nervous system. This may also provide a window of opportunity for DNA repair pathways to correct the damage. Neurons with defects in the ATM-MEF2 survival pathway are more sensitive and vulnerable to cell

death in the face of DNA damage. Patients with A-T exhibit persistent oxidative stress and reduced antioxidant capacity, whereas brains from ATM knockout mice show enhanced production of ROS and increased markers of oxidative stress (Reichenbach *et.al.*, 2002; Stern *et al*., 2002). Inappropriate response to DNA damage, increased oxidative stress and pro-survival signaling may eventually compromise neuronal survival. Failure of this pro-survival signaling cascade may thus contribute to the neurodegeneration in the cerebellum in A-T.

What would likely be the molecular basis and critical target gene for protection against cell death after DNA damage? Our group has identified multiple MEF2 binding sites located in the regulatory region of the Bcl-xl gene (unpublished observation). Bcl-xl represents one of the anti-apoptotic proteins of the Bcl-2 family and a key regulator of apoptosis in response to DNA damage (Boise *et al*., 1993; Frankowski *et al*., 1995; Gonzalez-Garcia *et al*., 1995). Hence, ATM-mediated phosphorylation/activation of MEF2D may drive the expression of Bcl-xl to protect neurons from DNA damage. A recent study has identified many MEF2 target genes that regulate neuronal survival and synaptic development (Flavell *et al*., 2008). One of the MEF2 target genes is brain-derived neurotrophic factor (BDNF), which is important for promoting neuronal survival. Activation of MEF2D by ATM may thus induce BDNF to potentiate neuroprotection from DNA damage. Further studies are needed to examine comprehensively the impact of the ATM-MEF2 pathway on the expression of MEF2 target genes.

In addition, prior studies have suggested that mitochondrial biogenesis is increased in response to DNA damage, and mitochondria are required for neuronal survival (Nicholls and Budd, 2000). Activation of ATM by etoposide has been reported

to upregulate the number of mitochondria (Fu *et.al*., 2008), and MEF2 plays an integral role in maintaining mitochondrial content, possibly via PGC1-α (Naya *et al*., 2002; Wu *et al*., 2002; Czubryt *et al*., 2003; Handschin *et al*., 2003). Accordingly, activation of MEF2 by ATM may conceivably protect neurons from DNA damage via maintenance of mitochondrial function.

Using protein sequence analysis, I have identified an ATM-interaction motif in MEF2D. ATM associates with MEF2D in response to DNA damage (Figure 3-16), and it will be of great interest to determine whether the physical interaction of ATM and MEF2D is mediated through this ATM-interaction motif or possibly via other specific regions in MEF2D. Site-directed mutagenesis will be needed to define the role of ATM-interaction motif in MEF2D for mediating ATM-MEF2D complex formation. A series of truncated MEF2D mutants will be used to determine whether other specific regions in MEF2D are essential for the association of ATM and MEF2D. In addition, future studies will be directed towards detecting ATM-MEF2D complex formation in the brain.

Previous study has shown that retinoic acid activates ATM and subsequently promotes differentiation of SY5Y neuroblastoma cells into neuronal-like cells (Fernandes *et al*., 2007). Our group recently used a constitutively active form of MEF2 under the regulation of the nestin enhancer to drive the differentiation of ESCs into neuronal progenitor cells (Li *et al*., 2008). Along this line, it will be interesting to investigate whether ATM-mediated phosphorylation/activation of MEF2D has a role for promoting neuronal differentiation under the same experimental conditions.

Previously, loss-of-function studies in MEF2D knockout mouse models have focused on heart development, and little is known about MEF2D function in the brain.

Because MEF2D knockout mice are viable, in the future it will be intriguing to investigate how DNA damage that is induced by IR or etoposide affects cerebellar granule cell neuronal survival in these mice *in vivo*. Such studies may provide further insight into the mechanism of this potential neuroprotective pathway *in vivo*. In addition, none of the ATM knockout mouse models show progressive neurodegeneration and ataxia similar to that found in human A-T (Barlow *et al*., 1996; Liyanage *et al*., 2000; Lavin *et al*., 2007). It will be attractive to examine whether the brain development of MEF2D knockout mice may recapitulate some neurological abnormalities of human A-T.

In summary, my results demonstrate that ATM-mediated phosphorylation and activation of MEF2D is essential for promoting neuronal survival in response to DNA damage. This new ATM-MEF2 pathway may provide a link between the DNA damage response and neuronal survival, and most importantly, should have therapeutic implications for promoting survival of cerebellar neurons in the brains of patients with A-T (Figure 4-1).

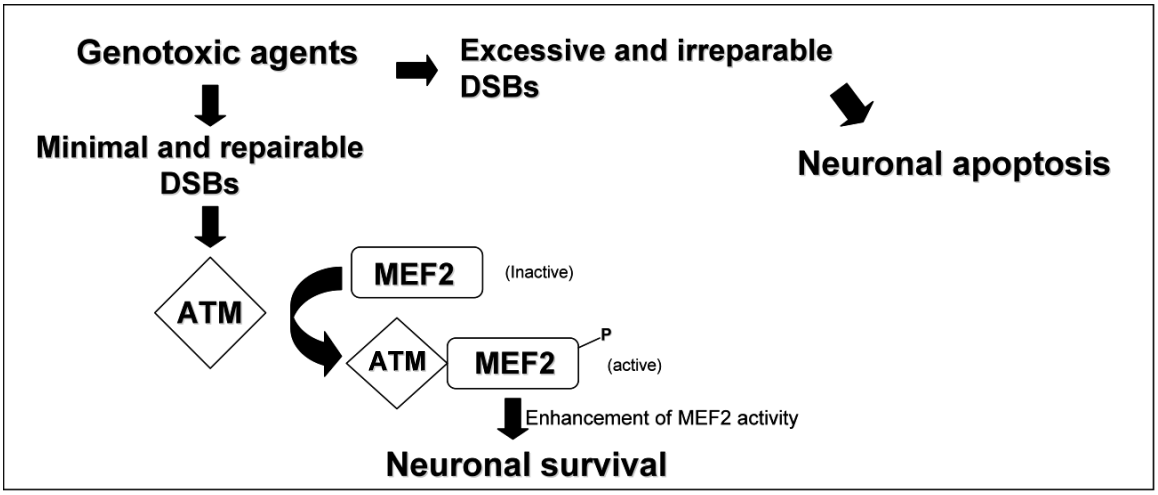

Figure 4-1. Proposed model for the role of ATM-dependent phosphorylation/activation of MEF2D for neuronal survival in response to DNA damage.

After DNA damaging agents cause double-strand breaks (DSBs) in exposed cells, ataxia telangiectasia mutated (ATM) is activated. In the face of minimal and repairable DNA damage, the activation of ATM can lead to i) formation of a complex of ATM and MEF2D and ii) phosphorylation and activation of MEF2. Potentiation of MEF2 activity via ATM phosphorylation can promote cell survival after DNA damage. In contrast, apoptosis predominates in the face of excessive and irreparable DNA damage.

Chapter 5: Materials and Methods

Primary cerebellar granule cell neuronal culture

All procedures involving animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Primary cultures of cerebellar granule cell neurons were obtained by mechanical and enzymatic dissociation of cerebella of postnatal day (P)6 or P7 day old Sprague-Dawley rats, ATM wild-type or knockout mice, as previously described (Levi *et al*., 1984). Briefly, animals were decapitated and cerebella were isolated and immersed in ice-cold HIB solution (120 mM NaCl, 5 mM KCl, 25 mM HEPES, 9.1 mM glucose). Dissociated cells were suspended in basal medium with Eagle's salts (BME), 25 mM KCl, 2 mM glutamine, and antibiotic (penicillin and streptomycin) and plated on poly-L-lysine coated dishes. At plating, 10% heat-inactivated FCS was added to the medium. After 36-48 h, 10 μM cytosine arabinoside was added to prevent nonneuronal cell replication. Thereafter, the medium was changed every two days until the cells were used for experiments. After 7-10 days in vitro (DIV), cerebellar granule cell neurons were used for experiments.

ATM knockout mice and genotyping

Mice deficient in ATM were provided by Dr. Carolee Barlow at Braincells Inc., La Jolla, California, U.S.A. Generation of animals and genotyping were done as previously described (Barlow *et al*., 1996). PCR-based genotyping was performed using the following primers:

1). ATM-forward (ATM-F): 5'-GACTTCTGTCAGATGTTGCTGCC-3',

2). ATM-back (ATM-B): 5'-CGAATTTGCAGGAGTTGCTGAG-3',

3). ATM-Neo: 5'-GGGTGGGATTAGATAAATGCCTG-3'.

cDNA constructs and plasmids

Expression plasmids for the GAL4 DNA binding domain [GAL4(DBD)] fused with the transactivation domains of human MEF2A [amino acids (aa) 87 to 505], human MEF2C (aa 87 to 442) and mouse MEF2D1b (aa 87 to 506) have been constructed as previously described (Han *et al*., 1997). His-tagged full-length MEF2A, -C and -D were cloned into the bacterial expression vector pETM1 by polymerase chain reaction (PCR) as previously described (Jiang *et al*., 1996; Han *et al*., 1997). Expression plasmids for wild-type and kinase-dead ATM were kindly provided by Dr. M. B. Kastan at St. Jude's Children's Research Hospital, Tennessee, U.S.A. The construct MEF2-dominant negative (MEF2-DN) retained the DNA-binding domain of MEF2 and acted as a dominant-interfering form because the transactivation domain was truncated at amino acid residue 105 and the remainder of the sequence was replaced with a Flag tag. The construct MEF2-constitutively active (MEF2-CA) contained a truncated version of the MEF2C transactivation domain but was constitutively active because it encoded a fully active VP16 transactivation domain (Okamoto *et al*., 2000).

I*n vitro* immunocomplex kinase assays

I*n vitro* immunocomplex kinase assays were carried out as previously described (Ziv *et al.*, 2000). Briefly, cell extracts from human embryonic kidney (HEK) 293T cells that have been transfected with 10 μg of wild-type ATM or kinase-dead ATM cDNAs

were prepared in modified TGN buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture from Roche Molecular Biochemicals and phosphatase inhibitor cocktail I and II from Sigma). Cleared supernatants were immunoprecipitated with an anti-Flag M2 antibody (Sigma) and protein A/G-agarose; the beads were washed with TGN buffer, followed by TGN buffer plus 0.5 M LiCl. Two additional washes with kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM $MnCl_2$) were performed. The immunoprecipitants were finally resuspended in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P] ATP, plus either 1 μg of recombinant GST-p53 or His-tagged MEF2A, -C, or -D fusion proteins. Kinase reactions were conducted at 30 °C for 20 min and stopped by the addition of SDS-PAGE loading buffer. Radiolabeled proteins were separated using SDS-PAGE and assessed with autoradiography. Transfection of kinase-dead ATM cDNAs was used as a control in these experiments. Protein loading levels were determined by Coomassie brilliant blue stains.

Preparation of recombinant His-tagged MEF2 fusion proteins

Escherichia coli BL21(DE3) was transformed with the vector pETM1 containing cDNAs encoding MEF2A, -C, or -D. The transformed bacteria were grown at 37 °C in LB broth until the A_{600} reached 0.5, at which time isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM for 5 h. Cells were collected by centrifugation at 8,000 *g* for 10 min, and the bacterial pellet was resuspended in 10 ml of buffer A (30 mM NaCl, 10 mM EDTA, 20 mM Tris-HCl, 2 mM phenylmethylsulfonyl

fluoride) for every 100 ml of original bacterial culture. Cell suspension was sonicated and cellular debris was removed by centrifugation at 10,000 *g* for 30 min. Recombinant proteins from the cleared lysate were purified using a Ni-nitrilotriacetic acid purification system (Qiagen).

Immunocytochemistry

Cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and then washed three times in PBS, permeabilized with 0.5 % Triton X-100 for 5 min, and washed three times. Nonspecific antibody binding was minimized by incubation in blocking solution (10 % goat serum in PBS) for 1 h at room temperature. Cells were incubated with primary antibodies against MEF2D (BD Transduction Laboratories) or phospho-(Ser/Thr) ATM/ATR substrates (phospho-SQ/TQ substrate antibody from Cell Signaling Technology) for overnight at 4 °C. After washing, cells were incubated with anti-mouse or anti-rabbit antibodies conjugated to FITC or Alexa 498/594 for 1 h at room temperature and then incubated in Hoechst 33342 dye (1 μg/ml) to fluorescently stain nuclei.

Immunostaining of ATM was performed as previously described (Wilson and Bianchi, 1999). Briefly, after chemical crosslinking was performed, cells were treated with PBS containing 1 % SDS for 5 min. Cells were incubated with a primary antibody against ATM (Abcam) for overnight at 4 °C. Control experiments with the secondary antibody alone were routinely performed and produced no detectable immunofluorescence signal (not shown).

DNA damaging agents and ATM inhibitor

Etoposide was obtained from Sigma-Aldrich. Cells were irradiated using a Gammacell 40 Exactor (MDS Nordion). The ATM inhibitor, KU-55933 was a kind gift from KuDOS Pharmaceuticals (Cambridge, UK).

Reporter gene assays

All luciferase assays were performed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. For GAL4-dependent luciferase reporter gene assays, the GAL4-responsive plasmid pG5E1bLuc, which contained five GAL4 sites cloned upstream of a minimal promoter driving a luciferase (luc) gene, was used as previously described (Gupta *et al*., 1995; Han *et al*., 1997). The reporter plasmids pG5E1bLuc and *Renilla* were co-transcfected into cells with a construct expressing GAL4-DNA binding domain (DBD) fused to MEF2A, -C, or -D, and along with an expression vector encoding wild-type ATM or kinase-dead ATM. Cells were grown on 35-mm-diameter multi-well plates and transiently transfected with 3 μg of total plasmid DNA using Lipofectamine reagent (Gibco BRL). *Renilla* expression plasmids were used to control for transfection efficiency. The total amount of DNA for each transfection was kept constant by using the empty vector pcDNA3. After 24 h, cell extracts were prepared, and luciferase activities were measured according to Promega protocol.

To measure endogenous MEF2 activity in cells, MEF2-dependent luciferase reporter gene assays were performed as previously described (Okamoto *et al*., 2000). The MEF2-dependent luciferase reporter gene was constructed by insertion of two

tandem copies of the MEF2 site from the brain creatine kinase enhancer upstream of the basal promoter of the embryonic myosin heavy-chain gene.

Assessment of apoptosis

To access apoptosis, the percentage of enhanced green fluorescent protein (EGFP)-positive cells with fragmented and condensed nuclei was assessed and quantified using fluorescence microscopy.

Immunoprecipitation (IP)

Cell lysates of HEK293T were prepared in NP40 or RIPA buffer with a protease inhibitor mixture (Roche Molecular Biochemicals) and phosphatase inhibitor cocktail I and II (Sigma) and immunoprecipitated by incubating at 4 ^{o}C for 90 min with protein A/G-agarose (1:1 slurry in NP40 or RIPA buffer) on a rocker. Following this pre-clearing step, the supernatants were centrifuged at 960 g for 3 min at 4 ^{o}C. Primary antibody was added at 10 μg/ml, incubated and rocked for 2 h at 4 ^{o}C. Protein A/G-agarose (1:1 slurry in NP40 or RIPA buffer) was added, and the mixture was incubated with rocking for 2 h at 4 ^{o}C. Following this incubation, the agarose was pelleted by centrifugation at 960 g for 3 min at 4 ^{o}C and washed three times with NP40 or RIPA buffer. After the final wash, the agarose was resuspended and boiled in 30 μl of 2x sample loading buffer at 100 ^{o}C for 7 min. IP samples were then analyzed by SDS-PAGE and Western blotting.

Western blotting

Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and were then blocked with 5 % nonfat dried milk in Tris-buffered saline containing 0.05 % Tween 20, pH 7.6 (TBST) for 1 h at room temperature. PVDF membranes were incubated with primary antibody in TBST containing 1 % nonfat milk for overnight at 4 °C. ATM-phosphorylated MEF2 proteins were detected with the phospho-SQ/TQ substrate antibody (Cell Signaling Technology). The antibody against MEF2D was purchased from BD Transduction Laboratories, and the anti-V5 antibody was purchased from Chemicon. Immunoreactive proteins were detected using anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase (Amersham Biosciences) and visualized with enhanced chemiluminescence by ECL kit (Amersham Biosciences).

RNA interference

psiSTRIKE-hMGFP vectors expressing shRNA targeting ATM, MEF2D and scrambled control (a control sequence that is not found in the mouse, human, or rat genome databases) were constructed according to the manufacturer's instructions (Promega). The following primers were used to generate shRNAs:

1). An shRNA-ATM-1: 5'-GCTGAGACAAATAATGTCT-3'

2). An shRNA-ATM-2: 5'-GGACAGGAATTACTTAAGT-3'

3). An shRNA-MEF2D-172: 5'-GCCAGCACCGACATGGACA-3'

4). An shRNA-MEF2D-176: 5'-GCACCGACATGGACAAGGT-3'

5). An shRNA-MEF2D-187: 5'-GACAAGGTGCTGCTCAAGT-3'

6). An shRNA-MEF2D-436: 5'-GTGCCCGTGTCCAATCAGA-3'

7). A scrambled shRNA: 5’-GCCTATTTCCCATGATTCC-3’

The following primers were used to generate shRNA resistant MEF2D cDNAs, and silent mutations were underlined:

1). An shRNA resistant MEF2D-1:

5’-GCCTGTCACGGTGCCAGTGTCCAATCAGAGC-3’

2). An shRNA resistant MEF2D-2:

5’-GCCTGTCACGGTGCCAGTCTCCAATCAGAGC-3’

3). An shRNA resistant MEF2D-3:

5’-GCCTGTCACGGTCCCAGTCTCCAATCAGAGC-3’

4). An shRNA resistant MEF2D-4:

5’-ACGGTCCCAGTCTCAAACCAGAGCTCACTG-3’

5). An shRNA resistant MEF2D-5:

5’-GTCCCAGTCTCAAACCAAAGCTCACTGCAG-3’

Cloning and mutation were confirmed by DNA sequencing.

Site-directed mutagenesis

Point mutations were introduced into wild-type MEF2D expression vector using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The following primers were used to generate a single mutation in MEF2D expression vector:

For mutation of threonine 259 into alanine,

T259A: 5’- ACCTACCCACAGCGCCCAGCTTGGAGCC-3'

For mutation of serine 275 into alanine,

S275A: 5’- CGAGTCATCACTGCCCAGGCAGGAAAGGGG-3'

For mutation of serine 294 into alanine,

S294A: 5’- CGCCTTGGGGTCGCCCAGTCTACTCATTCG-3'

For mutation of serine 314 into alanine,

S314A: 5’- AACGCCGAGTTTACTCGCCCAGGGCCTCCCC-3'

A combination of the above primers was used to generate triple and quadruple MEF2D mutants.

The following primers were used to generate the phospho-mimetic MEF2D mutants and nonphosphorylatable MEF2D mutants:

For mutation of threonine 259, serine 275, serine 294 and serine 314 into aspartic acid,

1). 5’-ACCTACCCACAGCGACCAGCTTGGAGCC-3'

2). 5’-CGAGTCATCACTGACCAGGCAGGAAAGGGG-3'

3). 5’-CGCCTTGGGGTCGACCAGTCTACTCATTCG-3'

4). 5’-GCCGAGTTTACTCGACCAGGGCCTCCCC-3'

For mutation of threonine 259, serine 275, serine 294 and serine 314 into glutamic acid,

1). 5’-ACCTACCCACAGCGAACAGCTTGGAGCC-3'

2). 5’-CGAGTCATCACTGAGCAGGCAGGAAAGGGG-3'

3). 5’-CGCCTTGGGGTCGAGCAGTCTACTCATTCG-3'

4). 5’-GCCGAGTTTACTCGAGCAGGGCCTCCCC-3'

Cloning and mutation were confirmed by DNA sequencing.

Statistics

All data are expressed as mean ± SD. Statistical evaluation was performed using analysis of variance (ANOVA) and Student’s *t*-test. Each experiment was performed ≥ 3 times in triplicate. Statistical analyses were performed with Prism 5.0 software (Graphpad) or Excel (Microsoft). $P < 0.05$ was considered statistically significant.

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