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IN VIVO SIGNIFICANCE OF THE MDM4 AND P73 INTERACTION DURING DEVELOPMENT AND TUMORIGENESIS

Mehrnoosh Tashakori

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IN VIVO SIGNIFICANCE OF THE MDM4 AND P73 INTERACTION
DURING DEVELOPMENT AND TUMORIGENESIS

by

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**IN VIVO SIGNIFICANCE OF THE MDM4 AND P73 INTERACTION
DURING DEVELOPMENT AND TUMORIGENESIS**

A
DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

BY

Mehrnoosh Tashakori, M.D.

Houston, Texas

August 2015

DEDICATION

To

My Father Kazem Tashakori

My Mother Mahshid Mansour

My brother Mehrdad Tashakori

For Their Unconditional Love and Endless Support

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ABSTRACT

IN VIVO SIGNIFICANCE OF THE MDM4 AND P73 INTERACTION DURING DEVELOPMENT AND TUMORIGENESIS

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The tumor suppressor protein p53 is negatively regulated by Mdm4 protein. The significance of such regulation was determined from mouse models. *Mdm4*-deficient mice are embryonic lethal at E7.5 in a *p53*-dependent manner. p73, a member of the p53-family, is a transcription factor with tumor suppressor activity. *In vitro* studies show that Mdm4 binds to p73 and, further, comprehensive biochemical studies revealed that Mdm4 has higher affinity for p73 than p53. However, little is known about the significance of the Mdm4 and p73 interaction *in vivo*. This study aimed to elucidate the biological consequences of this interaction during embryogenesis and tumorigenesis using genetic mouse models.

My study revealed that *p73* loss does not rescue the *Mdm4*-deficient embryonic lethality, indicating that unrestricted p53 activity leads to the

phenotype. Furthermore, loss of *p73* does not rescue the runted phenotype of *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos. These findings underscore that unrestricted p53 activity, even at the haploid level, suffices to cause embryonic lethality. Given the prominent roles of Mdm4 and p73 in developing brain, examining the importance of their interaction in this organ was noteworthy. *Mdm4*-deficiency in the brain causes porencephaly and late gestational embryonic lethality. This phenotype is rescued by deletion of *p53*. My work depicted that loss of *p73* does not rescue the porencephaly phenotype at E14.5. However, interestingly, in the absence of *Mdm4*, p73 is transcriptionally active and possibly contributes to the vigorous senescent and apoptotic phenotype of p53 in embryonic brain.

Since overexpression of *Mdm4* and loss of *p73* have been implicated in tumor development, I monitored a cohort of mice comprising *Mdm4*^{Tg15} *p73*^{+/-}, *Mdm4*^{Tg15}, *p73*^{+/-} and wild type mice. While no survival differences were observed, *Mdm4*^{Tg15} *p73*^{+/-} mice had increased incidence of lymphoma and brain tumors as well as advanced stage lymphoma with extensive dissemination compared to *Mdm4*^{Tg15} mice. These observations suggest that increased Mdm4 and p73 haploinsufficiency cooperate in tumorigenesis. Combined, this study suggests that the Mdm4-p73 axis is not as significant as the Mdm4-p53 pathway during embryogenesis and tumor development.

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CHAPTER 1. INTRODUCTION

The p53 protein family

The p53 protein family consists of three transcription factors, p53, p63 and p73. Having a common ancestor (1), the proteins in this family have substantial structural homology; each contains an N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD). Such homology results in functional similarities, as evidenced by induction of apoptosis and cell cycle arrest in response to DNA damage (2). However, these genes also have very distinct functions as a result of exposure to different selection pressures during evolution. While p53 functions as the guardian of genome and maintains genome integrity, both p63 and p73 play more prominent roles during development (1-3).

p53, the guardian of genome

Since its discovery in 1979, p53 has been extensively studied in the field of cancer research. p53, well known as the “guardian of genome” (3) and the “cellular gatekeeper” (4), is altered in the majority of human tumors (3). This alteration can be through mutation or deletion of the *p53* gene itself, or its functional inactivation via upregulation of its negative regulators, Mdm2 and Mdm4 (5).

p53 structure

The human *p53* gene is mapped to chromosome 17p13.1. It has 11 exons, which encode 393 amino acids for the full-length isoform of the p53 protein (6). Thus far, the structural analyses on p53 revealed that this protein has five main functional domains (Figure 1).

The N-terminal region of p53 (1-101 aa) consists of an acidic TAD and a proline-rich region (7). The TAD interacts with various proteins, such as components of transcription machinery (8, 9), the transcriptional coactivators such as p300/CBP (10, 11) and p53 negative regulators, MDM2 and MDM4 (12-14). The TAD is subdivided to two subdomains, TAD1 (amino acids 1-42) and TAD2 (amino acids 43-63) (7, 15). TAD1 has critical residues (Leu-22 and Trp-23) which determine the strength of its binding to the TATA-binding protein (TBP) and consequently control the rate of transcription (7). Moreover, these residues are necessary for MDM2 interaction with p53, which results in concealing of the TAD and inhibition of p53 activity (16). TAD2 also plays a role in activation of various p53 target genes (1); however, if TAD2 is mutated, TAD1 can compensate for normal transactivation of p53 (17).

The proline-rich region has PXXP motifs, suggestive of mediating protein-protein interactions. However, mouse models reveal that this region has structural rather than functional role. The complete deletion of proline-rich domain alters the p53 structure sufficiently to disrupt its function in mice, whereas mutations in this region are dispensable for tumor suppressor activity of p53 *in vivo* (18).

The central core of p53 (amino acids 102-292) comprises the DBD that recognizes the specific DNA sequence in the regulatory region of p53 target genes (19). Most cancer-related p53 mutations are found in this region, highlighting the significance of this domain in p53 tumor suppressor activity. These mutations alter either the ability of p53 to bind the target DNA sequence (DNA contact mutations) or the structure of the p53 DBD (conformational mutations) (20).

The C-terminal region of p53 contains the OD (also known as tetramerization domain) (amino acids 324-355) and a basic, lysine-rich domain (amino acids 356-393). Since p53 is active as a tetramer, the presence of tetramerization domain is essential for binding to DNA with high affinity (21). Moreover, the basic region is essential for p53 stability and its sequence-specific DNA binding (22).



Figure 1. Schematic representation of domain structure of p53. From the N-terminus to the C-terminus: transactivation domain 1 (TAD1), transactivation domain 2 (TAD2), the proline-rich (PR) region, the central DNA binding domain (DBD), the oligomerization domain (OD) and the basic, lysine-rich domain (Basic). Numbers at the bottom indicate the approximate position of residues at the beginning and the end of each domain.

p53 functions

In response to any cellular stress or damage that threatens the integrity of genome, p53 is activated via protein stabilization and post-translational modifications. Various stressors activate p53 in different ways. For instance, upon DNA damage due to ionizing radiation, p53 is activated by two kinases, ATM (Ataxia Telangiectasia mutated) and Chk2, whereas in response to oncogene activation such as Ras or Myc, p14ARF plays a role in stabilization of p53 (5). p53 tetramers bind to p53 response elements in the promoters of p53 target genes. These specific DNA sequences are classically defined as two copies of the 10 base pair motif RRRCWWGYYY (R: purine, W: adenine or thymine, Y: pyrimidine) separated by 0-13 base pairs (19). A myriad of genes have been identified to be p53 transcriptional targets and mediate various p53 functions, such as *p21*, *GADD45* and *14-3-3 σ* for cell cycle arrest, *p21*, *PAI1* and *PML* for senescence, *NOXA*, *PUMA* and *BAX* for apoptosis (5, 23-25) (Figure 2).

The fate of cells due to p53 activation depends on cell type, external environment, the type and level of stress. In response to irreparable damages, p53 induces irreversible apoptosis and senescence, whereas in minor insults, p53 mediates cell cycle arrest and DNA repair to allow cells to repair damage and revert back to normal state (23, 26). p53 mediates several other protective mechanisms to maintain genome stability, such as expression of antioxidants (27), inhibition of glycolysis as well as promotion of oxidative stress (28) and autophagy (29, 30) (Figure 2).

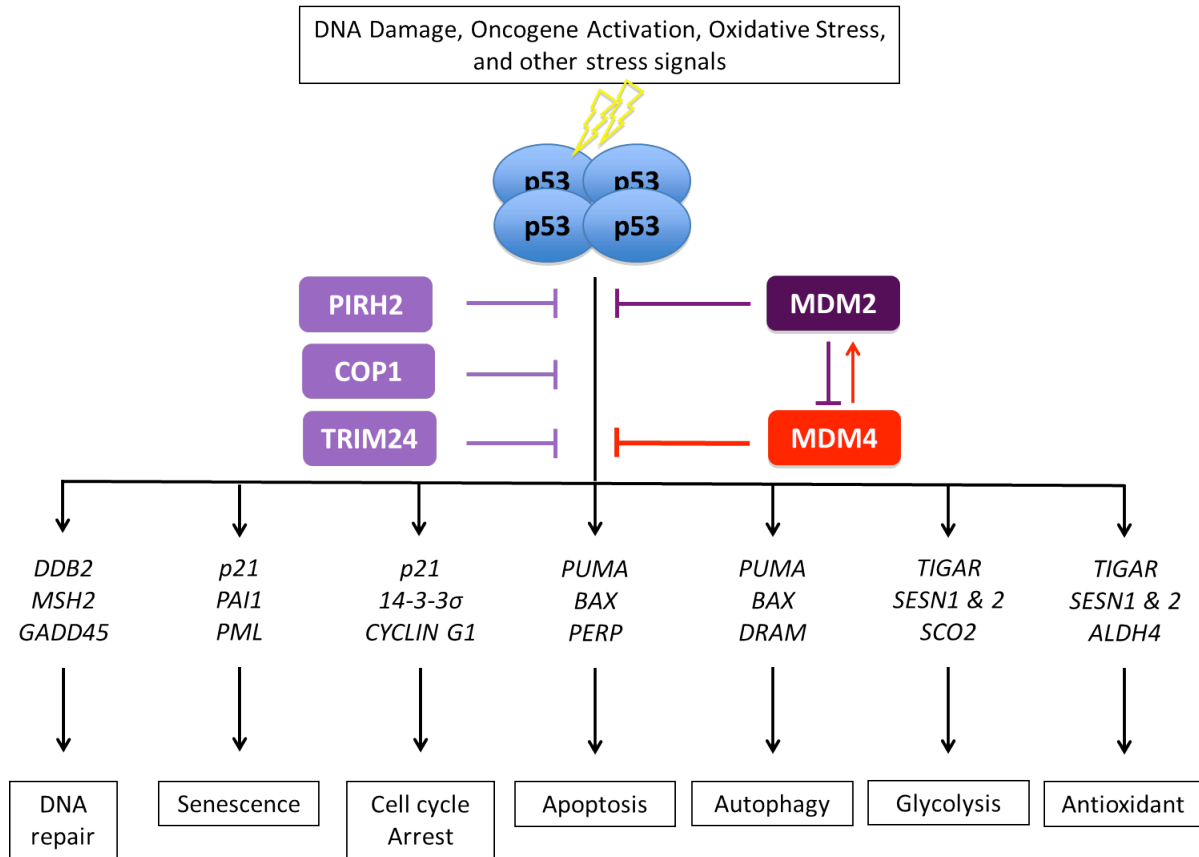


Figure 2. p53 functions. In response to a wide range of cellular stress, such as DNA damage, oncogene activation, and oxidative stress, p53 regulators stabilize and activate p53 through post-translational modification. Activated p53 exerts its tumor suppressor function via upregulation of a plethora of target genes involved in apoptosis, cell cycle arrest, senescence, autophagy, DNA repair and metabolism, to name but a few. In unstressed condition, several negative regulators have been identified that dampened p53 levels and activity, including MDM2, MDM4, PIRH2, COP1 and TRIM24, to name a few.

p53 regulators

As an unstable protein with the half-life of less than 20 minutes (31, 32), p53 levels in unstressed cells are controlled by the rate of its degradation (5). Several post-translational modifications contribute to p53 stability and activity, including but not limited to phosphorylation, acetylation, ubiquitination and sumoylation (33). For instance, under physiological conditions, Mdm2 binds to the N-terminus of p53 and mediates p53 degradation by ubiquitination. Upon stress, such as DNA damage, phosphorylation of multiple amino acids in the N-terminus of p53 results in dissociation of Mdm2 and recruitment of transcriptional coactivators such as p300/CBP. Acetylation of the C-terminus of p53 by p300/CBP further assures the exclusion of Mdm2 and the engagement of the other components of transcriptional machinery (11, 33).

Mdm2 is not the only enzyme which degrades p53. It has been shown that restored p53 in the *Mdm2*-null background degrades, albeit at a slower rate (34, 35). To date, a number of p53 regulators, which modulate p53 protein level, have been identified. For instance, Pirh2, Trim24 and Cop1, like Mdm2, are RING-type E3 ubiquitin ligases that regulate p53 levels through ubiquitination-mediated proteasomal degradation (Figure 2).

Another mechanism by which p53 is controlled under physiological conditions is via blocking of its TAD. Negative regulators such as Mdm2 and Mdm4 bind to the TAD of p53 and by physical masking of this domain inhibit p53 transcriptional activity (Figure 2).

Among all negative regulators of p53, genetic mouse studies highlight that Mdm2 and Mdm4 are master regulators of p53 at least during development and homeostasis, as evidenced by the early embryonic lethality of *Mdm2*- and *Mdm4*-deficient mice due to extensive p53 activity, a phenotype that was not observed upon deletion of other negative regulators of p53 (36-38).

Significance of p53 regulation and its gene dosage

Several studies and multiple mouse models have elucidated the importance of *p53* gene dosage during tumorigenesis and survival. A comprehensive comparison of various *p53* mouse models expressing different levels of p53 revealed that expression of only 7% of wild type p53 in *p53*^{neo/-} mice strikingly increases survival and shifts tumor spectrum compared to *p53*^{-/-} mice (39). *In vivo* genetic studies have shown that changes in the level of p53 regulators also affect the tumor phenotype. Both *Mdm2* and *Mdm4* transgenic mice develop spontaneous tumors and the main proposed oncogenic effect of these overexpressed proteins is through inhibiting p53 function (40, 41). The *Mdm2*^{SNP309G/G} mice harboring a regulatory polymorphism of the *Mdm2* gene also succumbs to tumors. This tumor-prone mouse model with slightly higher level of Mdm2 emphasizes how a subtle change in a negative regulator of p53 dampens the p53 pathway and increases tumor susceptibility (42). Conversely, deletion of one allele of *Mdm2* or *Mdm4* combined with transgenic expression of *Myc* oncogene or loss of tumor suppressor *Rb* extends survival of mice or decreases tumor burden, highlighting the protective effect of increasing p53 level (43-45).

Mdm4, the master regulator of p53

Mdm4 identification

Some years after identification of Mdm2 as a binding partner and inhibitor of p53 (46, 47), a cDNA clone was isolated through screening of 16-day-old whole mouse embryo cDNA expression library with radiolabeled p53 protein. Since the construct had a high homology to *Mdm2*, at both the DNA and protein level, it was called *Mdmx* (also known as *Mdm4*) (48). The co-expression of *p53* and *Mdm4* in mammalian cells followed by immunoprecipitation showed that the two proteins interact in cells. Moreover, this association inhibited the transcriptional activity of p53, indicating that Mdm4 most probably interacted with the N-terminal region of p53 (48). By fluorescent in situ hybridization (FISH), the *Mdm4* gene was later mapped to chromosome 1 (region F-G) (49). A year later, *MDM4*, the human homolog of *Mdm4*, was identified with 90% similarity to murine *Mdm4* and mapped to chromosome 1q32 (49). The expression analysis of both human and mouse *Mdm4* showed that they are ubiquitously expressed in all tissues. The highest level of Mdm4 is observed in thymus (48, 49).

Mdm4 structure

Human *Mdm4* gene is mapped in chromosome 1q32.1. It comprises 11 exons with the first ATG start codon in the second exon (48) and encodes MDM4 protein with 490 amino acids. Like Mdm2, Mdm4 protein has three conserved domains: p53-

binding domain at the N-termini, Acidic and Zinc-finger domains in the center and the Really Interesting New Gene (RING) finger domain at C-termini (Figure 3).

The p53-binding domains in both MDM proteins are highly homologous with 53.6% sequence similarity. Interestingly, the specific residues of this region, which are essential in p53 binding, are completely conserved in MDM2 and MDM4 (50). However, the X-ray crystallographic structures of TA domain of p53 and p53-binding domain of MDM4 depicted some variations between MDM2 and MDM4 binding to p53. For instance, the central hydrophobic cleft of MDM4 on which the TA domain of p53 binds is smaller due to protrusion of the side chains of two MDM4 residues (Met53 and Tyr99) into the binding pocket (51). The observation that Nutlin-3a, a small molecule that disrupts the MDM2-p53 interaction, has lower potency in abrogating the MDM4-p53 complex further highlights the difference between the p53-binding domains of the two MDM proteins.

Another domain with high homology (53.2%) between MDM2 and MDM4 is the C-terminal RING-finger domain. However, unlike MDM2, the MDM4 RING domain does not possess E3 ubiquitin ligase activity (14). Nonetheless, the MDM4 RING domain is essential for the Mdm2-Mdm4 heterodimerization (52), MDM2 stability and consequently p53 degradation by the MDM2 ubiquitin ligase activity (53, 54) (Figure 2). Interestingly, the MDM2-MDM4 hetero-RING complexes are more common than the MDM2 homo-RING complex *in vivo* and exert a higher E3 ubiquitin ligase activity (53). The importance of this domain is further strengthened by genetic mouse models. Disruption of the Mdm4 RING domain by either deletion or point mutation is embryonic lethal at E9.5. Both phenotypes are rescued by

concomitant deletion of p53, indicating the enhanced activity of p53 in the absence of MDM4 RING domain (32, 55).

Although MDM4, unlike MDM2, does not have a canonical nuclear localization signal (NLS), a potential NLS within the RING finger domain has been suggested that contains basic amino acids (RRLKK) and, upon DNA damage, plays a role in nuclear translocation of MDM4 (50). Another study has shown that p53 and Mdm2 also play roles in shuttling Mdm4 into the nucleus upon DNA damage (56).

The functions of two domain in the central region of MDM4, the zinc finger domain with high similarity to MDM2, and acidic domain with no significant homology to MDM2, remain largely unclear (57).

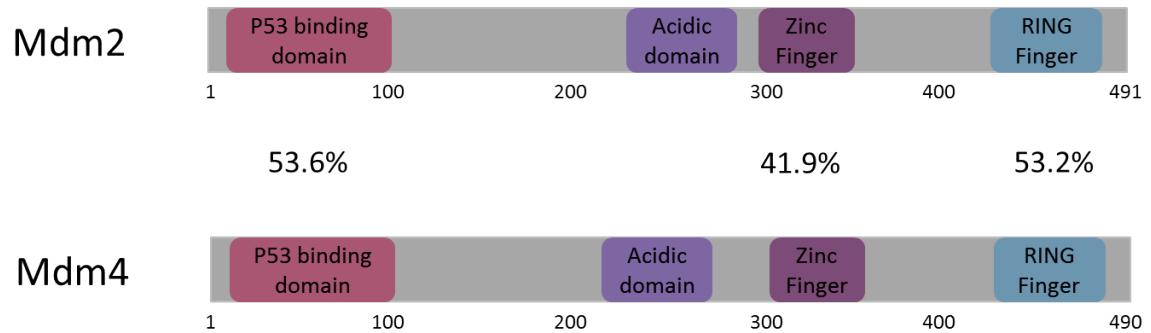


Figure 3. Schematic representation of Mdm2 and Mdm4 structure. The functional domains are highlighted. The p53-binding domain, zinc finger and RING finger domain are well conserved. The percentage homology shared between these domains is indicated.

Regulation of Mdm4

a. Regulation of Mdm4 at transcription level

Although Mdm4 is not induced by p53, recent studies have shown that Mdm4 expression correlates to Mdm4 promoter activity and mRNA levels (58). A cluster of transcription binding sites in the promoter of Mdm4 (c-Ets-1, Elk-1 and Aml-1) has been recognized to be critical for increased Mdm4 expression in tumor cell lines. Moreover, mitogenic signaling through the Ras/Raf/MEK/ERK signaling pathway regulates Mdm4 expression at the mRNA transcription level via increased binding of Est-1 and Elk-1 to the Mdm4 promoter (58). The observations that MDM4 protein levels correlate with mRNA level in tumor cells and that the half-life of Mdm4 protein does not increase upon using MG132 (proteasome inhibitor) in unstressed cells (59), signify that, under physiologic conditions, Mdm4 expression is controlled at the transcription level.

b. Regulation of Mdm4 at the post-transcriptional level

MDM4 has five alternative splicing variants, which have been studied in detail for their effect on p53 inhibition. It has been proposed that the ratio of these splice variants to the full-length *Mdm4* is of prognostic significance for cancer patients (60).

MDM4-S is an MDM4 truncated protein as a result of a deletion in exon 6 and creation of a stop codon in exon 7 which shifts the reading frame. Consequently, this variant contains only the p53-binding domain and is suggested to bind to p53 and inhibit its activity stronger than full-length MDM4 (61). The overexpression of MDM4-S transcript has been observed in primary soft tissue sarcomas and suggested as a

critical prognostic factor in these tumors (62). A subset of papillary thyroid carcinomas with wild type *p53* has also been found to have MDM4-S overexpression (63). Another MDM4 variant, MDM4-221, was also observed in this tumor group whereas, interestingly, the full-length MDM4 level was significantly decreased in these tumors (63).

The other two splicing variants, MDM4-A and MDM4-G, were identified in C233A cervical cancer cell lines. MDM4-A lacks the acidic domain due to deletion of exon 9 and MDM4-G lacks the p53-binding domain due to deletion of exons 3-6. Both forms inhibit p53 activity and stabilize MDM2, however, their physiologic function remains unclear (64).

MDM4-Alt1 and MDM4-Alt2 transcripts are produced at various doses and times in response to DNA damage. MDM4-Alt1, similar to MDM4-S, has the p53-binding domain and binds and represses p53 activity whereas MDM4-Alt2 lacks this domain and possibly stabilizes p53 by disrupting its binding to MDM2 (65).

c. Regulation of Mdm4 at the post-translational level

Several post-translational modifications have been characterized for MDM4 including phosphorylation and ubiquitination, which result in either activation or repression of p53 activity.

Phosphorylation of MDM4 at Ser367 by AKT kinases stabilizes MDM4, which in turn stabilizes MDM2 and inhibits p53 (66). Moreover, phosphorylation of MDM4 at S289 by Casein kinase 1 α (CK1 α) also results in inhibition of p53 by increasing MDM4 affinity for p53 (67). Upon genotoxic stress, c-Abl phosphorylates MDM4 at

Y99, which causes a conformational change that impairs the MDM4-p53 interaction and thus activates p53 (68).

In response to DNA damage, the C-terminal of MDM4 is phosphorylated by ataxia-telangiectasia mutated (ATM) at S403 (69) and Checkpoint kinase 2 (Chk2) at S342 and S367 (70). Such phosphorylation in or close to the RING domain, along with other DNA damage-induced modifications of MDM2, reduce the MDM4 affinity for deubiquitylating enzyme HAUSP (71, 72) and leads to ubiquitination and degradation of MDM4 by MDM2 (73). Consequently, upon MDM4 degradation, MDM2 is destabilized and degraded and p53 is activated.

MDM4 is also SUMOylated at K254 and K379 (74); however, the significance of these modifications remains unclear.

d. Regulation of Mdm4 by Localization

Under physiologic conditions, Mdm4 is localized in the cytoplasm and cooperates with MDM2 to inhibit p53 activity by promoting cytoplasmic localization of p53 (75). Upon DNA damage, p53 and MDM2 induce MDM4 nuclear translocation through complex formation. Further, nuclear MDM4 regulates p53 by blocking MDM2-mediated nuclear export of p53 and inhibiting MDM2-mediated p53 ubiquitination (56, 76). In addition, a fraction of MDM4 localizes at the mitochondria and acts as a scaffold for the p53 and Bcl-2 interaction and, consequently, promotes apoptosis (77).

Mdm4 regulatory functions

a. Significance of Mdm4 in regulating of p53 and lessons from animal models

Similar to Mdm2, Mdm4 is a *bona fide* regulator of p53 during embryonic development, DNA damage and physiological conditions. The *Mdm2*-null mice die at embryonic day (E) 3.5 prior to implantation while *Mdm4*-deficient mice arrest at pre-gastrulation and die at E7.5. These embryonic lethal phenotypes are due to apoptosis and cell cycle arrest, respectively, and both are completely rescued by deletion of *p53* (78-80).

Multiple tissue-specific and cell type-specific studies identified distinct and independent roles of Mdm2 and Mdm4 in regulating p53. While *Mdm2* loss in cardiomyocytes is embryonic lethal at E13.5 due to apoptosis (80), mice with *Mdm4*-deficient cardiomyocytes are viable but have a shortened life span (median survival of 234 days) due to apoptosis in cardiomyocytes and consequent dilated cardiomyopathy (81). Deletion of either *Mdm2* or *Mdm4* in neural progenitor cells of mouse is embryonic lethal. However, their phenotypes and p53-dependent mechanisms are different. *Mdm2* loss in the brain results in hydranencephaly (absence of the brain's cerebral hemispheres) due to apoptosis, whereas *Mdm4* deficiency causes porencephaly (presence of a cavity or cyst within the cerebral hemisphere) due to both apoptosis and cell cycle arrest. Both phenotypes are lethal at E12.5 and E17.5, respectively, and rescued by deletion of *p53*. Interestingly, concomitant deletion of *Mdm2* and *Mdm4* in the developing CNS resulted in a more severe phenotype compared to loss of each gene alone, revealing that these proteins are not functionally redundant (82). Interestingly, Mdm2 overexpression can

compensate for Mdm4 deficiency and rescue the embryonic lethal phenotype by dampening p53 activity (83).

In addition, while *Mdm2*^{+/-} or *Mdm4*^{+/-} mice have a normal life span, both are radiosensitive and, upon DNA damage, succumb to p53-dependent early death (43). On the other hand, *Mdm2*^{+/-} *Mdm4*^{+/-} double-heterozygous mice are not viable due to neural and hematopoietic defects. This phenotype is rescued by loss of a single *p53* allele, underscoring the fine-tuning in the p53/Mdm2/Mdm4 regulatory network and the cell-type specific sensitivity (43). Moreover, conditional deletion of *Mdm2* in adult mice is also lethal due to extensive p53-induced irreversible tissue damage (34, 84), whereas deletion of *Mdm4* under similar condition is compatible with life (85).

b. p53-independent functions of Mdm4

Although the main known function of Mdm4 is inhibition of p53 activity, Mdm4 was found to have p53-independent roles. *Mdm4*^{-/-} *p53*^{-/-} double null mice have shorter survival compared to *p53*^{-/-} mice and chromosomal studies on derived tumor cells as well as MEFs reveal that Mdm4 indeed suppresses multipolar mitotic spindle formation and chromosome loss independent of p53 and Mdm2 (86). Additionally, elevated levels of Mdm4 delay DNA damage response and enhance genome instability and transformation. These functions are also independent of p53 and Mdm2 (87). Combined, these studies underscore the importance of Mdm4 levels *per se* in maintaining genomic stability.

Additionally, Mdm4 interacts with p21, promotes its proteasomal degradation in an ubiquitin-independent process and, consequently, abrogates G₁ cell-cycle

arrest. p21 degradation by Mdm4 is independent of, but cooperates with Mdm2 (88). E2F-1 is another protein that Mdm4 binds to and represses its transactivation independent of Mdm2 and p53. Unlike the p21-Mdm4 interaction, Mdm4 does not alter E2F-1 protein level, but reduces its ability to bind to its consensus DNA sequence (89) or increases cytoplasmic localization of E2F-1 (90).

Role of Mdm4 in tumorigenesis and lessons from animal models

Many human tumors have high level of MDM4 due to either gene amplification or overexpression. For instance, elevated levels of MDM4 are detected in retinoblastoma (91, 92), melanoma (93), colon cancer (58, 94), breast cancer (94, 95), Ewing sarcoma (96), lung cancer (94), prostate cancer (97), glioblastoma (98) and hematopoietic neoplasms such as pre-B acute lymphoblastic leukemia (ALL) in adults (99) and acute myeloid leukemia (AML) (100).

Given that many tumors have high levels of MDM4 and that MDM4 is a critical regulator of p53, it was postulated that MDM4 overexpression *in vivo* would be tumorigenic. In fact, three independent lines of *Mdm4* transgenic mice develop spontaneous tumors in which the *Mdm4* transgene is driven by the cytomegalovirus (CMV) immediate-early (IE) enhancer and the chicken β -actin promoter, allowing ubiquitous expression of Mdm4 (41). In addition, this Mdm4 transgenic mouse in the background of p53 heterozygosity also accelerates tumorigenesis and shows a distinct tumor spectrum from mice with either genetic change alone, highlighting the cooperation of Mdm4 and p53 during tumor development (41).

The Mdm4-p53 axis, a targetable pathway for cancer therapy

The major role of MDM4 in tumor development and progression is to inhibit p53 transcriptional activity. Interestingly, a large number of the tumors with high MDM4 retain wild type *p53*. Mdm4 exerts its inhibitory effect via physical masking of the transactivation domain of p53 and unlike Mdm2, has no role in controlling the level of p53 protein by degradation. Accordingly, mouse studies showed that deletion of *Mdm2* in adult mice causes detrimental p53-mediated tissue damage and death (34, 84), whereas deletion of *Mdm4* under comparable conditions only results in minor reversible damages (85). Thus disruption of the Mdm4-p53 interaction and restoration of wild type 53 activity has been proposed as a druggable target and tested as an anti-cancer therapy in mouse models. Interestingly, using Nutlin-3, a small molecule inhibiting the p53-Mdm2 interaction as well as the p53-Mdm4 interaction with lower affinity, combined with Topotecan in retinoblastoma xenograft model (91) enhances sensitivity to cytotoxic agents and synergistically kills tumor cells. Moreover, SAH-p53-8, a stapled peptide interrupting the MDM-p53 interaction, combined with Cisplatin in melanoma cell lines also results in higher tumor cell death compared to treatment with chemotherapeutic agent alone (93). Such data encourage combination therapy and adding Mdm4 inhibitors to the therapeutic regimen of tumors with high expression of Mdm4 and wild type p53.

p73, the guardian's elder brother

p73 was discovered approximately 2 decades after p53 and named “the long lost cousin of p53” due to its substantial degree of structural and functional homology to p53. Since then, various in-depth and detailed studies have shown that despite the similarities, p73 has differences in structure as well as function. The structural analyses suggest that p73 is the ancestor of p53 and, for this reason it is also called “the guardian's elder brother” (101). Moreover, the functional studies showed that this gene has remarkable roles during development.

p73 Structure

The human *p73* gene maps to chromosome 1p36.3, a region frequently deleted in a subset of human cancers. It has 14 exons with the first coding sequence in exon 2. Compared to *p53*, *p73* has larger introns and a few more exons. The larger size of introns permits a higher level of recombination and greater diversity of haplotypes.

p73 has high structural homology to p53. Similar to p53, p73 has an N-terminal TAD with 30% homology to p53, a DBD with the highest sequence similarity to p53 (63%) (2) and an OD with 38% identity (2, 102) (Figure 4). Having the highest homology in the DBD as well as identical residues that interact with DNA, allows p73 to bind to the consensus p53-binding site, activate common target genes and mediate apoptosis and cell cycle arrest (103). On the other hand, p73 and p53 have the most prominent structural differences in the C-terminus. While p53 has

approximately 30 amino acids at the C-terminus that interact with DNA nonspecifically, p73 has more than 200 amino acids, which includes a sterile-alpha motif (SAM) domain (a protein-protein domain) (104). Most proteins with SAM domains have a regulatory role during development (105); this supports the notion that p73 is involved in differentiation and development.

Shortly after its identification, the *p73* gene was characterized to have a dual structure with a distal and internal promoter in intron-3 as well as at least seven alternatively spliced C-terminal isoforms (α , β , γ , δ , ϵ , ζ , η). At that time, only one full-length isoform was known for p53, a notion that changed thereafter (106, 107). The distal promoter leads to the expression of a full-length TAp73 isoform with transactivation activity, whereas the internal promoter leads to expression of the $\Delta Np73$ isoform, an N-terminally truncated protein lacking the transactivation domain (107) (Figure 4).

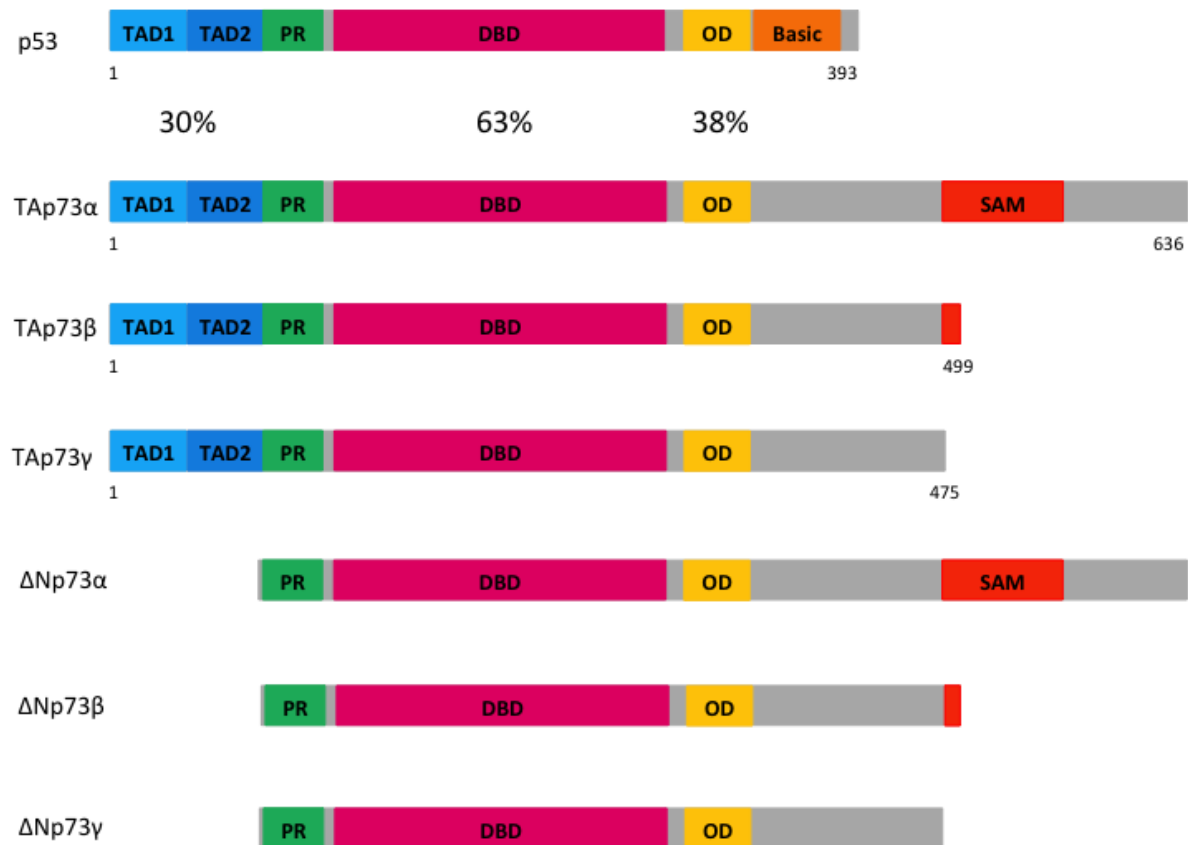


Figure 4. Schematic representation of p73 structure and comparison of domain structure of p53 and p73. p53 and p73 share similarity in TAD, DBD and OD. The percentage identity shared between these domains is indicated. p73 has two main isoforms: The TAp73 isoform, a full-length protein with TAD at N-terminus, and the ΔNp73 isoform, an N-terminally truncated protein lacking TAD. P73 also has alternatively spliced C-terminal isoforms such as α, β and γ. All these isoforms have the DNA binding domain (DBD) and the oligomerization domain (OD), and some such as TAp73α and ΔNp73α have extra domains, for instance sterile-alpha motif (SAM) domain.

p73 regulators

Similar to p53, the stability and activity of p73 are regulated through protein-protein interactions and subsequently post-translational modifications, such as ubiquitination, phosphorylation and acetylation (108, 109).

In response to genotoxic stress, one of the first modifications of p73 is phosphorylation by a non-receptor tyrosine kinase, c-Abl, at Tyr99, Tyr121 and Tyr240 (110), which leads to p73 interaction with other proteins such as PIN1, and acetylation by p300 and eventually its stabilization and activation (111, 112). Moreover, c-Abl activates JNK/p38 MAPK pathway, which in turn phosphorylates p73 at threonine residues close to proline and promotes its accumulation (113). c-Abl also phosphorylates YAP, which further associates with p73 and promotes p73 transcriptional activity (114).

p73 is also phosphorylated during cell cycle by cyclin-dependent kinases (CDKs) in unstressed cells. Phosphorylation of p73 at threonine 86 in the by S/G₂/M CDK complexes decreases p73 transcriptional activity and enables cell cycle progression (115).

Similar to p53, the level of p73 is mainly regulated by the ubiquitin-proteasome system. Itch, a HECT-domain E3 ubiquitin ligase, was the first enzyme identified to target p73 for proteasomal degradation (116). Two proteins, N4BP1 and YAP, modulate this process by competing with Itch in their binding to p73 (117, 118).

p73 functions

Given that p73 has various isoforms with distinct functions, the overall activity of p73 depends on the ratio of its different isoforms.

a. Tumor suppressor activity of TAp73

1. Growth arrest

The TAp73 isoform, which contains the N-terminal TAD, suppresses tumorigenesis by mediating cell cycle arrest and apoptosis as well as maintaining genome stability. Similar to p53, TAp73 activates *p21*, *GADD45* and *14-3-3 σ* to induce cell cycle arrest in G1 and G2/M (119-123). Furthermore, it regulates the number of cell divisions by repressing human telomerase reverse transcriptase (hTERT) expression. However, the mechanism is controversial: some studies suggest this is through Sp1, whereas others propose it is through NF-YB2 (124, 125).

2. Genomic stability

p73 inhibits polyploidy and aneuploidy through regulation of G2-M damage checkpoints in a p53-independent manner (126). Moreover, upon DNA double-strand breaks, p73 regulates DNA repair through upregulation of *Brca2* and *Rad51*, genes involved in homologous recombination (HR) DNA repair pathway (127). More detailed studies on isoforms revealed that TAp73-deficient MEFs and oocytes have mitotic spindle defects and premature mitotic exits (128). Further studies showed that the interaction of TAp73 with BubR1 in the spindle assembly checkpoint (SAC) complex is essential for proper mitosis and lack of this isoform leads to dysfunctional

SAC and consequently aberrant mitosis and aneuploidy (129). Moreover, p73 coordinates mitotic exit via *p57* transactivation (130) and mediates mitotic cell death through *Bim* upregulation (131).

3. Apoptosis

Upon stress, TAp73 induces cell death through various pathways. It regulates mitochondrial-mediated apoptosis through transactivation of p53 target genes such as Bcl-2 family members *Bax*, *Puma* and *Noxa* or via activation of *Gramd4* in a p53-independent manner (132-134). p73 also plays a role in the extrinsic apoptosis pathway by stimulating expression of CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 (134, 135). Moreover, TAp73 induces endoplasmic reticulum stress by upregulating *Scotin* in response to DNA damage (134).

b. Oncogenic activity of Δ Np73

Since Δ Np73 does not have the TA domain, it is unable to induce gene expression and consequently does not mediate growth arrest or cell death. However, this isoform impacts these functions by its dominant-negative effect on TAp73 and p53. Δ Np73 isoforms oligomerize with TAp73 isoforms or compete with p53 on binding to p53 target genes to compromise their transcriptional function. Intriguingly, both TAp73 and p53 induce the expression of Δ Np73, forming a feedback loop to fine-tune the tumor suppressor activity of TAp73 and p53 (136, 137). Therefore, the ratio and activity of TAp73 and Δ Np73 isoforms determine the cell fate. Shifting the balance towards Δ Np73 favors cancer progression by inhibiting the growth arrest and death of tumor cells as well as enhancing the tumorigenic

phenotype by $\Delta Np73$ gain of function properties, such as chemoresistance, induction of EMT and metastasis (109, 136, 138).

Role of p73 during development and lessons from animal models

One distinct difference between p53 and p73 is their role during development. While *p53*-null mice are for the most part viable without prominent developmental defects, *p73*-deficient mice have multiple developmental abnormalities. These mice are runted with a high mortality rate (139). However, the severity depends on the background of mice. More than 75% of *p73*^{-/-} mice in the SJ129 background die in less than a month, whereas *p73*^{-/-} mice in a mixed genetic background survive longer (107). The cause of the early death is usually gastrointestinal hemorrhage or, less commonly, intracranial bleeding (139).

Surviving *p73*^{-/-} mice acquire generalized pan-mucositis and chronic bacterial infections, which are characterized by massive inflammation and neutrophil aggregation. However, no obvious deficiencies were identified in lymphoid or granulocyte populations, indicating that the phenotype is mainly related to the surface barriers of innate immune system such as epithelial lining of the mucosa and secreted mucosa and cytokines (2, 139). Moreover, *p73* deficiency causes abnormalities in pheromonal signaling pathways, which results in lack of sexual interest and infertility in mature *p73*^{-/-} mice (139). In addition, *p73*^{-/-} mice display severe neurological defects including the core triad of *p73* deficiency in the brain: cortical hypoplasia, hippocampal malformation and ventriculomegaly (139, 140). This phenotype varies in severity: the severe form has massive apoptosis and

causes early postnatal death, whereas the mild form has none or low levels of apoptosis and half of the pups might survive to adulthood (140).

p73 has a role in corticogenesis as early as E12.5, as evidenced by ectopic neurons in *p73*-deficient preplate and cortical hem (141), and the decrease in cortical thickness becomes apparent after E14.5, ranging from 10-30% compared to wild type brain at the same developmental stage. It seems that hypoplasia of the cortical plate is due to defect in neural stem cells (140). At this stage, the size of ventricles is normal. However, ventriculomegaly and subsequently hydrocephalus happen shortly after birth and the severity is also variable. Hydrocephalus is a communicating form, indicating a defect in production or reabsorption of cerebral spinal fluid (CSF) due to loss of *p73* in ependymal cells (139, 140). In addition, the *p73*^{-/-} hippocampus has 3 characteristics: absence of Cajal-Retzius (CR) cells, lack of hippocampal fissures and dysgenesis of dentate gyrus (2, 139, 141). CR cells are early born neurons in the marginal zone of the developing cortex. These cells secrete Reelin, which is essential for radial migration of neurons (141, 142). Absence of these cells in *p73*^{-/-} mice causes architectonic abnormalities in the hippocampus, characterized by unusual arrangement of the CA1-CA3 pyramidal cell layer and lack of inferior blade of dentate gyrus (139, 141). Moreover, absence of hippocampal fissure indicates the significance of p73 in cortical folding (141).

A more detailed characterization of the phenotype of *p73*-deficient mice was learned from genetic studies on two p73 major isoforms, TAp73 and Δ Np73. Like *p73*^{-/-} mice, *TAp73*^{-/-} female and male mice are infertile due to abnormal mating behavior. Additionally, *TAp73*^{-/-} female mice ovulate fewer oocytes compared to wild

type females and among those ovulated ones, some are retained under the bursa of ovary (128). Moreover, *TAp73*^{-/-} male mice show increased DNA damage and cell death in spermatogonia as well as defective spermatids, which result in hypospermia and infertility (143). On the contrary, loss of $\Delta Np73$ does not interfere with fertility (144). Moreover, the only neurological defect of *TAp73*^{-/-} mice is hippocampal defect comparable to *p73*^{-/-} hippocampal abnormality (128), while $\Delta Np73$ ^{-/-} mice have reduced cortical thickness and decreased neuronal density in old age, compatible with neurodegeneration, compared to wild type mice at the same age (144). Such neuroanatomical alterations along with behavioral changes suggestive of Alzheimer's disease has been reported in the aged *p73*^{+/-} mice (145).

Role of p73 in tumorigenesis and lessons from animal models

Unlike *p53*, *p73* is mutated in less than 0.5% of human tumors (136). However, several studies have shown that *p73* is altered in various tumor types through loss of heterozygosity (LOH) or epigenetic silencing, including different types of lymphoma and leukemia (146-149), glioma (150), neuroblastoma (151), ovarian cancer (152), breast cancer (153) and colorectal cancer (154). Such observations emphasize *p73* tumor suppressor activity.

In fact, genetic studies clearly highlight this function as *p73*^{+/-} mice have significantly shorter life span compared to wild type mice and develop spontaneous thymic lymphoma, lung adenocarcinoma and hemangiosarcoma (155). Moreover, the combination of *p73* loss with loss of other tumor suppressor genes such as *p53* or the presence of an oncogene such as *Myc* enhance tumor burden and/or increase

tumor dissemination or metastasis (155-157). To elaborate in more detail, the median age of $p53^{+/-} p73^{+/-}$ mice is 6 months compared to 10 months in $p53^{+/-}$ mice. Furthermore, $p53^{+/-} p73^{+/-}$ mice have 50% frequency of metastasis compared to 0-5% in $p53^{+/-}$ mice (155, 158, 159). Loss of one allele of $p73$ in the $p53$ null background does not have a significant effect on the survival, whereas $p53^{-/-} p73^{-/-}$ mice have 11.5 days shorter life span compared to $p53^{-/-}$ mice. However, both $p53^{-/-} p73^{+/-}$ and $p53^{-/-} p73^{-/-}$ mice have higher rates of T-cell lymphoma (82% and 83%, respectively) compared to $p53^{-/-}$ mice (66%). More importantly, more than 75% of lymphomas in $p53^{-/-} p73^{+/-}$ and $p53^{-/-} p73^{-/-}$ mice are moderately to severely disseminated; whereas, only 10% of $p53^{-/-}$ lymphomas are moderately infiltrated to other organs (156). In $E\mu$ -myc transgenic mice overexpressing *Myc* in B cells, loss of $p73$ does not affect survival; however, it strikingly promotes the dissemination of lymphoma. The $p73^{-/-} E\mu$ -myc mice have massive infiltration of lymphoma to lung, meninges, brain parenchyma and paravertebral ridges compared to $E\mu$ -myc mice with localized lymphoma in lymph nodes and spleen (157).

$TAp73^{-/-}$ mice have a shorter life span compared to wild type mice and approximately 70% of $TAp73^{-/-}$ and 30% of $TAp73^{+/-}$ mice in a mixed background succumb to tumorigenesis with lung adenocarcinomas and lymphomas being the most prevalent cancers, respectively (128). On the other hand, $\Delta Np73^{-/-}$ mice have a normal life span and E1A/Ras^{V12} transformed $\Delta Np73^{-/-}$ MEFs develop significantly smaller tumors compared to wild type MEFs, underscoring that $\Delta Np73$ deficiency impairs tumor formation *in vivo* (144). Collectively, the genetic *in vivo* studies support the notion that the TAp73 isoform functions as a tumor suppressor inducing cell

cycle arrest and apoptosis while the Δ Np73 isoform inhibits such transactivation activity in a dominant-negative manner (137, 160, 161).

Interaction of Mdm4 with p73

Given the significance of Mdm2 and Mdm4 in regulating p53 stability and activity *in vivo* and the high structural homology of p53 and p73 in the TA domain, several studies examined the interaction of Mdm proteins with p73. Interestingly, the co-expression of *Mdm2* and *p73* human embryonic kidney (HEK-293) cells with wild type p53 as well as *p53*-deficient human osteosarcoma (Saos-2) and lung adenocarcinoma (H1299) cells showed that similar to the p53-Mdm2 interaction, p73 and Mdm2 bind via their N-termini (162-164). These *in vitro* studies revealed that such binding reduces p73 transcriptional activity (163, 165) and in turn *p73*-induced apoptosis (162). However, p53 and p73 bind to separate regions on Mdm2 (165) and, unlike p53, p73 is not destabilized or degraded by Mdm2 (162, 163). Moreover, recently, an *in vivo* study proposed that Mdm2 exerts its oncogenic activity through inhibiting both p53 and p73 functions (166).

Mdm4, like Mdm2, can also bind to p73 (164); yet, whether such interaction represses p73 transcriptional activity remains to be elucidated. Interestingly, a detailed quantitative characterization of interaction of Mdm proteins with p53-family proteins has revealed that Mdm4 has higher affinity for p73 than p53 (167). To interrogate the biological significance of the Mdm4-p73 interaction *in vivo*, here, I present a comprehensive genetic characterization of the potential Mdm4 and p73 interaction during both development and tumorigenesis.

CHAPTER 2. RESULTS

Loss of *p73* does not rescue the *Mdm4*-null embryonic lethality

Mdm4-deficient mice die at E7.5 due to unrestricted p53 activity. This embryonic lethality is rescued by loss of *p53*. Since *in vitro* studies showed that Mdm4 interacts with p73, and, more interestingly, Mdm4 binds to p73 with higher affinity compared to p53, I asked whether the phenotype of *Mdm4* deficiency is rescued by *p73* loss and examined the phenotype of *Mdm4*^{Δ2/Δ2} *p73*^{-/-} mice. I first crossed *p73*^{+/-} mice with *Mdm4*^{+/-} mice to generate breeders with desirable genotype, *Mdm4*^{+/-} *p73*^{+/-} mice. Next, I intercrossed *Mdm4*^{+/-} *p73*^{+/-} mice to obtain *Mdm4*^{Δ2/Δ2} *p73*^{-/-} embryos with the expected frequency of 6.25% or 1 in 16 mice.

Mdm4^{Δ2} allele is the null allele of *Mdm4* which is generated by deletion of exon 2, the first coding exon, and no Mdm4 protein was detected by western blot in *Mdm4*^{Δ2/Δ2} *p53*^{-/-} mouse embryonic fibroblasts (MEFs) (80). The *p73* null allele is generated by the replacement of exon 5 and 6 with neomycin-resistant (*Neo*^R) gene, and it does not have transcriptional activity due to the disruption of DNA-binding domain (139). *Mdm4* is on mouse chromosome 1 and *p73* is on chromosome 4, so these crosses were feasible. To overcome the issue of *p73*^{-/-} infertility, I used *p73* heterozygous mice as breeders in each cross.

Rescue study at E11.5

I intercrossed $Mdm4^{+/Δ2} p73^{+/-}$ mice and first examined the rescue at mid-gestation. At E11.5, I sacrificed pregnant females and collected 36 embryos with normal morphology, 2 resorbed embryos and 16 empty deciduae. I did not obtain any $Mdm4^{Δ2/Δ2} p73^{-/-}$ embryo with normal morphology; however, one of the two resorbed embryos was $Mdm4^{Δ2/Δ2} p73^{-/-}$ and the other was $Mdm4^{Δ2/Δ2}$ (Table 1). The phenotypes of both were comparable, indicating that $p73$ loss does not rescue $Mdm4^{Δ2/Δ2}$ phenotype at this developmental stage.

Rescue study at E9.5

Further, I studied the rescue at an earlier time point. I sacrificed pregnant females at E9.5 and obtained 37 embryos with normal morphology as well as 5 resorbed embryos. The normal embryos were wild type or heterozygous for $Mdm4$, whereas the resorbed embryos were $Mdm4$ -null with various $p73$ alleles (one $Mdm4^{Δ2/Δ2}$ (1), $Mdm4^{Δ2/Δ2} p73^{+/-}$ (1) and $Mdm4^{Δ2/Δ2} p73^{-/-}$ (3) embryos) (Table 2). Additionally, I observed no significant difference in the morphology of $Mdm4^{Δ2/Δ2} p73^{-/-}$ compared to $Mdm4^{Δ2/Δ2}$.

Combined, all the $Mdm4^{Δ2/Δ2}$ embryos, regardless of $p73$ genotype, were remnants of resorbed embryo with no recognizable morphology (Table 3) ($\chi^2=30.000$, $df=5$, $p\text{-value} < 0.0001$), indicating that loss of $p73$, in the presence of unrestricted p53 activity, does not rescue the $Mdm4$ -null phenotype.

Table 1. *p73* loss does not rescue the *Mdm4*-null embryonic lethality at E11.5

Cross: *Mdm4*^{+/ Δ 2} *p73*^{+/-} × *Mdm4*^{+/ Δ 2} *p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/+}	1	2.25	2.8	6.25
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/-}	7	4.5	19.4	12.5
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{-/-}	0	2.25	0	6.25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/+}	4	4.5	11.1	12.5
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/-}	14	9	38.9	25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{-/-}	10	4.5	27.8	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/+}	0 *	2.25	0	6.25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/-}	0	4.5	0	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{-/-}	0 *	2.25	0	6.25
Total	36 ¶	36	100	100

* *Mdm4* ^{Δ 2/ Δ 2} embryos are arrested at pre-gastrulation at E7.5. In total, one *Mdm4* ^{Δ 2/ Δ 2} *p73*^{+/+} and one *Mdm4* ^{Δ 2/ Δ 2} *p73*^{-/-} remnants of resorbed embryos were collected at E11.5.

¶ Sixteen empty deciduae were also collected.

Table 2. *p73* loss does not rescue the *Mdm4*-null embryonic lethality at E9.5

Cross: *Mdm4*^{+/ Δ 2} *p73*^{+/-} × *Mdm4*^{+/ Δ 2} *p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/+}	0	2.3125	0	6.25
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/-}	4	4.625	10.81	12.5
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{-/-}	7	2.3125	18.92	6.25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/+}	2	4.625	5.4	12.5
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/-}	18	9.25	48.65	25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{-/-}	6	4.625	16.22	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/+}	0 *	2.3125	0	6.25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/-}	0 *	4.625	0	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{-/-}	0 *	2.3125	0	6.25
Total	37	37	100	100

**Mdm4* ^{Δ 2/ Δ 2} embryos are arrested at pre-gastrulation at E7.5. In total, one *Mdm4* ^{Δ 2/ Δ 2} *p73*^{+/+}, one *Mdm4* ^{Δ 2/ Δ 2} *p73*^{+/-} and three *Mdm4* ^{Δ 2/ Δ 2} *p73*^{-/-} remnants of resorbed embryos were collected at E9.5.

Table 3. *p73* loss does not rescue the *Mdm4*-null embryonic lethality

Cross: *Mdm4*^{+/ Δ 2} *p73*^{+/-} \times *Mdm4*^{+/ Δ 2} *p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/+}	1	4.5625	1.369	6.25
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{+/-}	11	9.125	15.068	12.5
<i>Mdm4</i> ^{+/+} <i>p73</i> ^{-/-}	7	4.5625	9.59	6.25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/+}	6	9.125	8.219	12.5
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{+/-}	32	18.25	48.836	25
<i>Mdm4</i> ^{+/Δ2} <i>p73</i> ^{-/-}	16	9.125	21.918	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/+}	0 *	4.5625	0	6.25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{+/-}	0 *	9.125	0	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p73</i> ^{-/-}	0 *	4.5625	0	6.25
Total	73	73	100	100

**Mdm4* ^{Δ 2/ Δ 2} embryos are arrested at pre-gastrulation at E7.5. In total, two *Mdm4* ^{Δ 2/ Δ 2} *p73*^{+/+}, one *Mdm4* ^{Δ 2/ Δ 2} *p73*^{+/-} and four *Mdm4* ^{Δ 2/ Δ 2} *p73*^{-/-} remnants of resorbed embryos were collected at E11.5 and E9.5.

¶ Sixteen empty deciduae were also collected.

Gene dosage study to determine if *p53* haploinsufficiency contributes to the rescue of the *Mdm4*^{Δ2/Δ2} *p73*^{-/-} phenotype

I further examined the contribution of both *p53* and *p73* to the rescue of *Mdm4*^{Δ2/Δ2} early embryonic lethality. I set up crosses to determine whether the *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{+/-} or *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{-/-} mice are viable or developmentally more advanced compared to *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos.

Since *p53* is on mouse chromosome 11, it was possible to generate triple heterozygous *Mdm4*^{+/-Δ2} *p53*^{+/-} *p73*^{+/-} mice. I then intercrossed *Mdm4*^{+/-Δ2} *p53*^{+/-} *p73*^{+/-} mice to generate the breeders with desirable genotypes, *Mdm4*^{Δ2/Δ2} *p53*^{-/-} *p73*^{+/-} and *Mdm4*^{Δ2/Δ2} *p53*^{-/-} mice.

Gene dosage study at P7

I then crossed *Mdm4*^{Δ2/Δ2} *p53*^{-/-} *p73*^{+/-} with *Mdm4*^{+/-Δ2} *p73*^{+/-} mice to obtain *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{+/-} or *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{-/-} mice with the expected probability of 25% (1 out of 4 mice) and 12.5% (1 out of 8 mice), respectively. I analyzed 27 pups at postnatal day 7 (P7) and I found that neither *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{+/-} nor *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{-/-} pups are born alive (Table 4) ($\chi^2=27.667$, df =5, *p*-value< 0.0001). I therefore performed a developmental study for possible rescue.

Table 4. *p73* is haploinsufficient to rescue the *Mdm4*^{Δ2/Δ2}*p53*^{+/-} phenotype at P7

Cross: *Mdm4*^{Δ2/Δ2}*p53*^{-/-}*p73*^{+/-} × *Mdm4*^{+/-}*p73*^{+/-}

Genotype	Number of pups		Percentage of pups	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	6	3.375	22.22	12.5
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	13	6.75	48.15	25
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	8	3.375	29.62	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	0	3.375	0	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	0	6.75	0	25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	0	3.375	0	12.5
Total	27	27	100	100

Gene dosage study at E11.5

Given that $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ mice are not viable (79), I further examined the role of $p53$ gene dosage in the rescue of $Mdm4^{\Delta2/\Delta2}$ early embryonic lethality and the effect of $p73$ loss on the phenotype of $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ embryos. I crossed $Mdm4^{\Delta2/\Delta2} p53^{-/-}$ with $Mdm4^{+/Δ2} p73^{+/-}$ mice to test whether 50% reduction in $p53$ gene dosage extends the lifespan of $Mdm4^{\Delta2/\Delta2}$ embryos and concomitantly whether deletion of one allele of $p73$ suffices to further advance the partial rescue. At E11.5, I observed 10 $Mdm4^{+/Δ2} p53^{+/-}$ embryos with normal morphology as well as 17 $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ embryos with an aberrant developmental phenotype, suggesting that deletion of one allele of $p53$ cannot completely rescue the $Mdm4^{\Delta2/\Delta2}$ phenotype. However, decreased $p53$ dosage in $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ embryos did lead to a partial rescue of $Mdm4$ -null embryonic lethal phenotype. Although these embryos were still severely runted compared to $Mdm4^{+/Δ2} p53^{+/-}$ embryos (Figure 5a-c), they overcame pre-gastrulation arrest and developed further, as evidenced by the formation of a neural groove with neural folds on each side as well as the allantois, a conspicuous landmark of gastrulation (168). The observation of partial rescue due to haploid loss of $p53$ is in agreement with findings from another $Mdm4$ knockout mouse model, which is lethal at later developmental stage (E10.5) (169). Moreover, $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ and $Mdm4^{\Delta2/\Delta2} p53^{+/-} p73^{+/-}$ embryos had comparable phenotypes, indicating that loss of one allele of $p73$ is not sufficient to rescue $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ phenotype (Table 5) ($\chi^2 = 10.000$, $df = 3$; p -value = 0.0186).

Table 5. Loss of one allele of *p73* does not rescue the *Mdm4*^{Δ2/Δ2}*p53*^{+/-} phenotype at E11.5

Cross: *Mdm4*^{Δ2/Δ2}*p53*^{-/-} × *Mdm4*^{+/-Δ2}*p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/-Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	5	2.25	50	25
<i>Mdm4</i> ^{+/-Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	5	2.25	50	25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	0 §	2.25	0	25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	0 §	2.25	0	25
Total	10 ¶	10	100	100

§ At E11.5, nine *Mdm4*^{Δ2/Δ2}*p53*^{+/-}*p73*^{+/+} and 8 *Mdm4*^{Δ2/Δ2}*p53*^{+/-}*p73*^{+/-} runted embryos were observed. All *Mdm4*^{Δ2/Δ2}*p53*^{+/-} embryos, regardless of *p73* genotype, were comparable morphologically.

¶ Eight empty decidua were also collected.

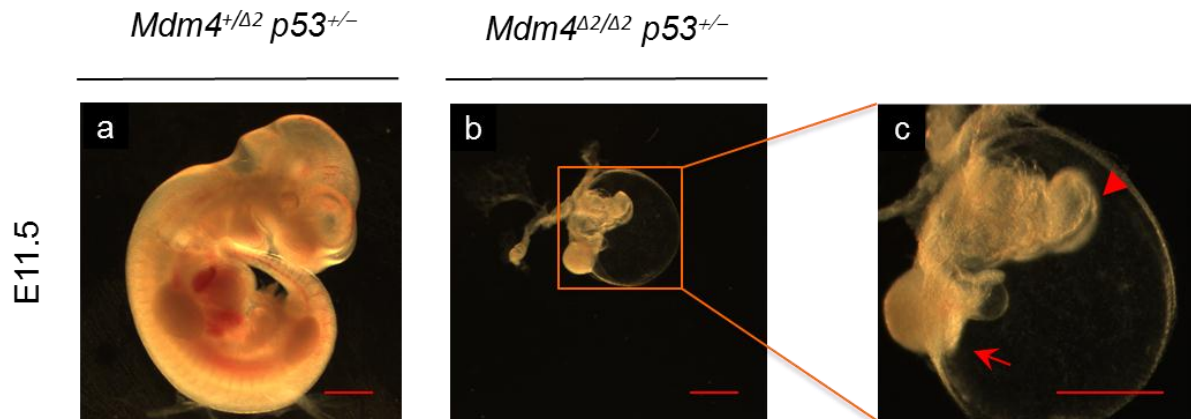


Figure 5. Representative pictures of the gross morphology of $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ embryos at E11.5. (a) $Mdm4^{+/Δ2} p53^{+/-}$ embryo, (b,c) $Mdm4^{\Delta2/\Delta2} p53^{+/-}$: The arrowhead points to the neural fold and the arrow points to the allantois. This phenotype was consistent among all $Mdm4^{\Delta2/\Delta2} p53^{+/-}$ embryos, regardless of $p73$ genotype. Capturing the picture of the remnant of resorbed $Mdm4^{\Delta2/\Delta2}$ embryos was not possible due to technical issues. Bar: 1 mm

Gene dosage study at E9.5

To determine whether the complete loss of *p73* can extend the survival of *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos, I crossed *Mdm4*^{Δ2/Δ2} *p53*^{-/-} *p73*^{+/-} with *Mdm4*^{+/-} *p73*^{+/-} mice and analyzed embryos at an even earlier time point. At E9.5, I obtained 18 *Mdm4*^{+/-} *p53*^{+/-} embryos, which were morphologically comparable to wild type, and 28 *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos, which were abnormally small (Table 6) ($\chi^2=30.000$, $df=5$, $p\text{-value} < 0.0001$). The *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos in the background of wild type, heterozygous or null *p73* allele had the same phenotype (Figure 6), suggesting that the unrestricted haploid level of p53 is strong enough to cause embryonic lethality.

Table 6. *p73* loss does not prolong the partial rescue of *Mdm4*^{Δ2/Δ2}*p53*^{+/-} embryos at E9.5

Cross: *Mdm4*^{Δ2/Δ2}*p53*^{-/-}*p73*^{+/-} × *Mdm4*^{+Δ2}*p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	1	2.25	5.55	12.5
<i>Mdm4</i> ^{+Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	14	4.5	77.78	25
<i>Mdm4</i> ^{+Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	3	2.25	16.67	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	0 §	2.25	0	12.5
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	0 §	4.5	0	25
<i>Mdm4</i> ^{Δ2/Δ2} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	0 §	2.25	0	12.5
Total	18 ¶	27	100	100

§ At E9.5, seven *Mdm4*^{Δ2/Δ2}*p53*^{+/-}*p73*^{+/+}, 16 *Mdm4*^{Δ2/Δ2}*p53*^{+/-}*p73*^{+/-} and 5 *Mdm4*^{Δ2/Δ2}*p53*^{+/-}*p73*^{-/-} developmentally retarded embryos were observed.

¶ Six empty deciduae were also collected.

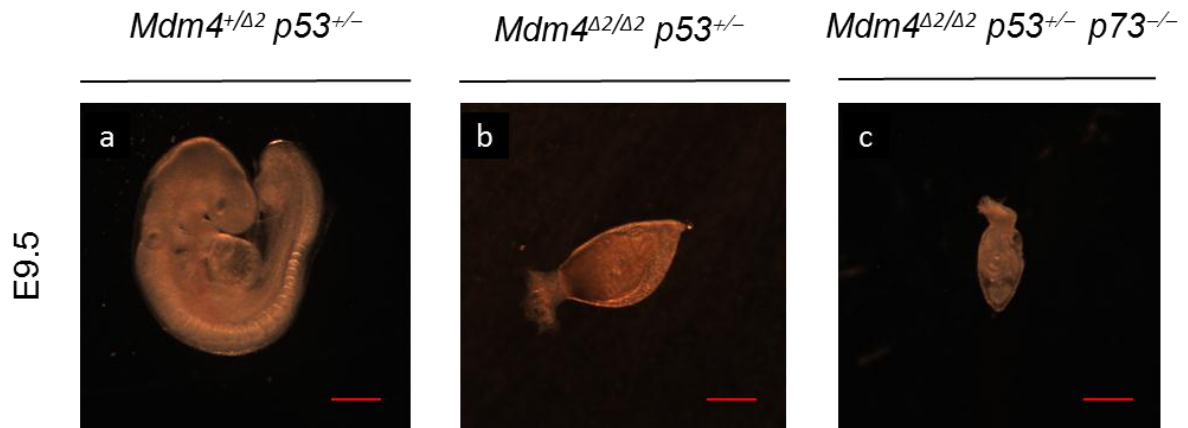


Figure 6. Representative pictures of the gross morphology of *Mdm4*^{Δ2/Δ2} *p53*^{+/-} embryos at E9.5. (a) *Mdm4*^{+/Δ2} *p53*^{+/-}, (b) *Mdm4*^{Δ2/Δ2} *p53*^{+/-}, (c) *Mdm4*^{Δ2/Δ2} *p53*^{+/-} *p73*^{-/-} embryos. Capturing the pictures of the remnant of resorbed *Mdm4*^{Δ2/Δ2} and *Mdm4*^{Δ2/Δ2} *p73*^{-/-} embryos were not possible due to technical issues. Bar: 1 mm

Loss of *p73* does not rescue the *Mdm4*^{Δ2/Δ2} embryonic brain phenotype

Next, I focused on the possible cooperation of Mdm4 and p73 during development of the brain, an organ wherein both Mdm4 and p73 have well-established role during embryogenesis. Additionally, such organ-specific approach would circumvent widespread p53-mediated early embryonic lethality of *Mdm4* deficiency.

Mdm4 plays an important role in neural development by regulating p53 (169). Conditional deletion of *Mdm4* in the developing CNS by *Nestin*-driven *Cre recombinase* (*Nes-cre*) starting as early as E10.5 (82, 170) results in porencephaly, a cavity in the cerebrum, and pups are not viable after birth due to severe brain-tissue deficiency. This phenotype is a gradually progressive neuronal loss due to both cell cycle arrest and apoptosis and completely rescued by deletion of *p53* (82).

On the other hand, p73 is well known as a multifunctional protein in the field of neurobiology (171) and crucial for maintenance of neural stem cells (172, 173). p73 is expressed sparsely in preplate, the first stage in corticogenesis, as early as E10.5, and it is highly expressed throughout the cortical hem and in CR cells at E12.5 (139, 141). However, the phenotype of *p73*-deficiency in the brain is not evident during prenatal life, except mild cortical hypoplasia after E14.5. The core triad of neurological defects in *p73*^{-/-} mice, including cortical hypoplasia, hippocampal dysgenesis and ventriculomegaly, is only prominent in postnatal and adult mice (140).

Given that both Mdm4 and p73 has established roles in the development of embryonic brain, we tested whether Mdm4 is a negative regulator of p73 during CNS

development and examined if the phenotype of *Mdm4* deficiency in the CNS is rescued by *p73* loss. In this study, I used the *Mdm4*^{fx} allele in which the floxed exon 2 is deleted upon expression of Cre recombinase under control of *Nestin* promoter and neuron specific enhancer (82, 170). Such Cre-mediated recombination is highly efficient and specific (82).

We crossed *Mdm4*^{fx/fx} *p73*^{+/-} mice with *Mdm4*^{+/-} *p73*^{+/-} *Nes-cre* mice to generate *Mdm4*^{fx/fx} *p73*^{-/-} *Nes-cre* embryos. Embryos were collected at E14.5, the earliest time point that apoptosis and cell cycle arrest due to lack of *Mdm4* are apparent in the embryonic brain (82). In total, 121 embryos were collected (Table 7). The first 48 embryos were fixed in 4% paraformaldehyde and among those, the ones with the following genotype were subjected to histopathological studies: *Mdm4*^{+/-} and *Mdm4*^{fx/fx} (wild type embryonic brain), *Mdm4*^{+/-} *p73*^{-/-} and *Mdm4*^{fx/fx} *p73*^{-/-} (*p73*^{-/-} embryonic brain), *Mdm4*^{fx/fx} *Nes-cre* (*Mdm4*^{Δ2/Δ2} embryonic brain), and *Mdm4*^{fx/fx} *p73*^{-/-} *Nes-cre* (*Mdm4*^{Δ2/Δ2} *p73*^{-/-} embryonic brain). The midline sagittal sectioning revealed that the phenotype of porencephaly in all three *Mdm4*^{fx/fx} *Nes-cre* embryos and seven *Mdm4*^{fx/fx} *p73*^{-/-} *Nes-cre* embryonic brains was comparable (Figure 7).

Moreover, *Mdm4*^{fx/fx} *p73*^{-/-} *Nes-cre* brains, similar to *Mdm4*^{fx/fx} *Nes-cre*, had decreased proliferation and increased apoptosis rates, as evidenced by Ki67 and cleaved caspase-3 (CC3) staining, and there was no difference in the pattern of proliferation and apoptosis between the two genotypes (Figure 8). These results clearly show that even in a tissue wherein *p73* has prominent functions, disrupting the *Mdm4*-*p73* interaction is not a major cause of the *Mdm4*-null phenotype.

Table 7. *p73* loss does not rescue the *Mdm4*-deficiency brain phenotype

Cross: *Mdm4*^{+/*fx*} *p73*^{+/-} *Nes-cre* × *Mdm4*^{*fx/fx*} *p73*^{+/-}

Genotype	Number of embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{+/+}	5	7.5625	4.1	6.25
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{+/+} <i>Nes-cre</i>	6	7.5625	5	6.25
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{+/-}	9	15.125	7.4	12.5
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{+/-} <i>Nes-cre</i>	18	15.125	14.9	12.5
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{-/-}	8	7.5625	6.6	6.25
<i>Mdm4</i> ^{+/<i>fx</i>} <i>p73</i> ^{-/-} <i>Nes-cre</i>	11	7.5625	9.1	6.25
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{+/+}	10	7.5625	8.3	6.25
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{+/+} <i>Nes-cre</i>	10 [§]	7.5625	8.3	6.25
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{+/-}	11	15.125	9.1	12.5
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{+/-} <i>Nes-cre</i>	15 [§]	15.125	10.7	12.5
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{-/-}	4	7.5625	3.3	6.25
<i>Mdm4</i> ^{<i>fx/fx</i>} <i>p73</i> ^{-/-} <i>Nes-cre</i>	14 [§]	7.5625	13.2	6.25
Total	121	121	100	100

[§]All embryos displayed porencephaly.

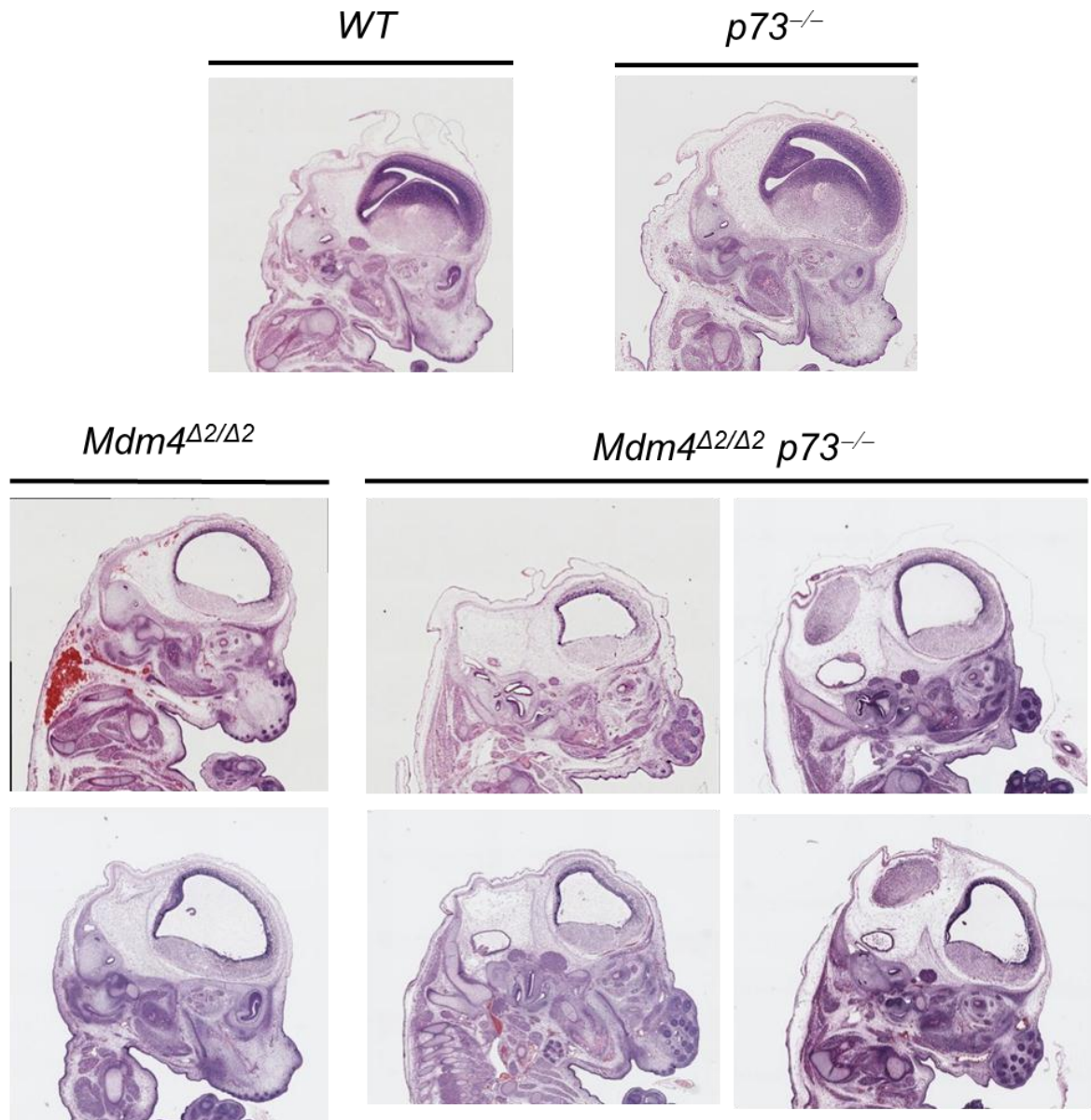


Figure 7. Loss of *p73* does not rescue the *Mdm4*-null porencephaly phenotype. Representative pictures of the sagittal sections of embryos at E14.5. The genotype of brain is mentioned on each column. Sections are stained with H&E.

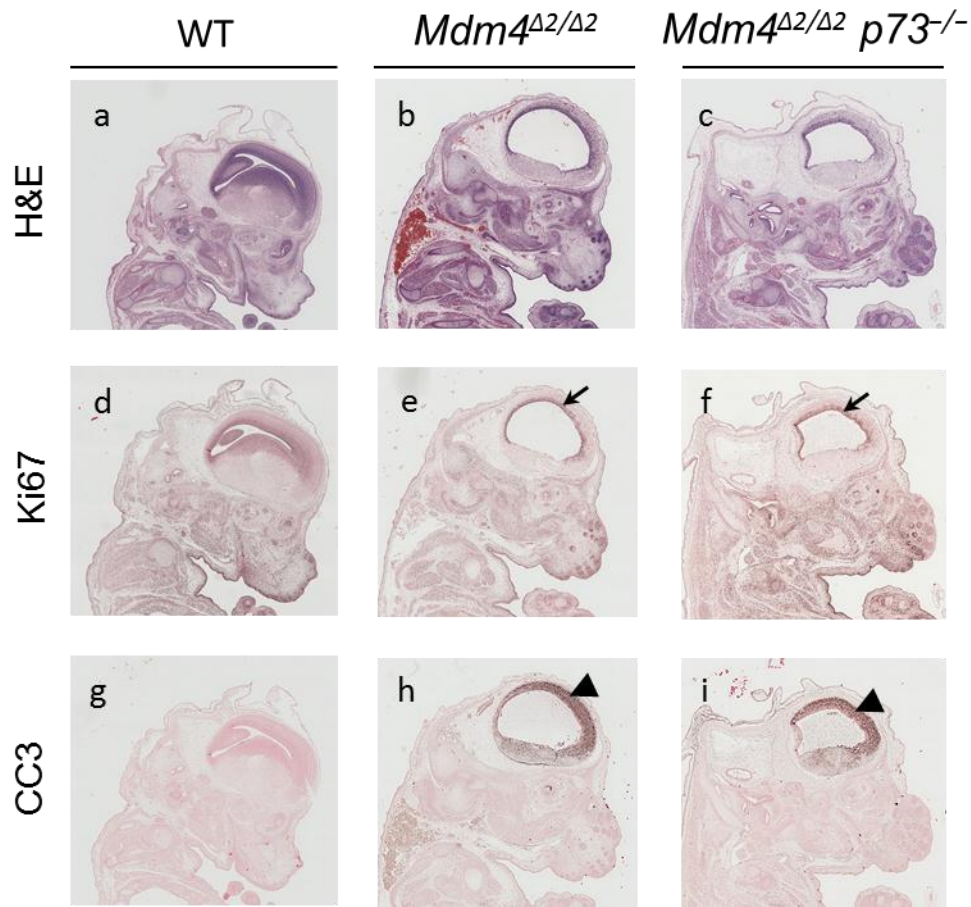


Figure 8. Loss of *p73* does not affect the pattern of proliferation and apoptosis in the *Mdm4*-null brain phenotype. Representative sagittal sections of embryos at E14.5, the genotype of brain is mentioned in each column. Sections are stained with H&E (a-c) or subjected to immunohistochemistry for Ki67 (d-f) as a marker of proliferation or cleaved caspase-3 (g-i) as a marker of apoptosis. The arrow points to positive Ki67 cells and the arrowhead points to positive CC3 cells.

Mdm4 inhibits the expression of some p73 and p53 target genes in the embryonic brain

Given the strong evidence of the Mdm4-p73 interaction *in vitro*, we decided to interrogate the inhibitory regulation of Mdm4 on p73 activity at the molecular level in our system. Several studies have been performed to identify the p73 specific target genes in various cell types or cancer cell lines under different conditions. In each set of experiments, different target genes have been identified (121, 122, 127, 173). Thus, we decided to examine, in our experimental model system, genes identified as p73 targets and known to play a role in the developing CNS or adult brain. Since p53 and p73 have the highest homology in their DNA binding domain compared to other domains (174), it is not surprising that a number of candidate genes are in fact common p53 and p73 target genes.

Although the phenotype of *Mdm4* deficiency was apparent at E14.5, there was sufficient brain tissue remaining for RNA isolation. Thus, 73 embryos were collected at this time point and their brains were dissected and preserved in TRIzol. The embryos with the following genotypes were used for RNA isolation and subsequently gene expression analysis by real-time RT-qPCR: *Mdm4*^{+/*fx*} and *Mdm4*^{*fx*/*fx*} (wild type embryonic brain), *Mdm4*^{+/*fx*} *p73*^{-/-} and *Mdm4*^{*fx*/*fx*} *p73*^{-/-} (*p73*^{-/-} embryonic brain), *Mdm4*^{*fx*/*fx*} *Nes-cre* (*Mdm4* ^{$\Delta 2/\Delta 2$} embryonic brain), *Mdm4*^{*fx*/*fx*} *p73*^{-/-} *Nes-cre* (*Mdm4* ^{$\Delta 2/\Delta 2$} *p73*^{-/-} embryonic brain). The *Mdm4* ^{$\Delta 2/\Delta 2$} *p53*^{-/-} embryonic brains at E14.5 were obtained from intercrossing *Mdm4* ^{$\Delta 2/\Delta 2$} *p53*^{-/-} mice.

p53 and p73 common target genes

14-3-3 σ

14-3-3 σ is a p53 target gene, which inhibits G2/M progression and induces cell cycle arrest (175). Overexpression of p73 upregulates the transcript of 14-3-3 σ two to six times higher than by does p53 overexpression (121, 122) and that p73 directly binds to the consensus p53/p73-binding site in the regulatory region of 14-3-3 σ gene (121), suggesting that 14-3-3 σ might be a *bona fide* target of p73. Interestingly, a comprehensive study on the differential expression of 14-3-3 protein isoforms showed that 14-3-3 σ is located in the nuclei of developing rat hippocampus neurons (176) as well as human hippocampus (177), corresponding to the region that p73^{-/-} and TAp73^{-/-} mice display brain phenotypes.

To examine the inhibitory effect of Mdm4 on p73 in the developing CNS, I asked whether deletion of *Mdm4* in the embryonic brain upregulates 14-3-3 σ transcript levels. As control, I used *Mdm4* ^{$\Delta 2/\Delta 2$} p73^{-/-} and *Mdm4* ^{$\Delta 2/\Delta 2$} p53^{-/-} embryonic brains. Indeed, 14-3-3 σ was highly expressed in *Mdm4* ^{$\Delta 2/\Delta 2$} embryonic brain compared to wild type (*p-value* < 0.01) and deletion of p73 or p53 abrogates such transcriptional upregulation (*p-value* < 0.01 and <0.05, respectively) (Figure 9), indicating that both p53 and p73 regulate 14-3-3 σ .

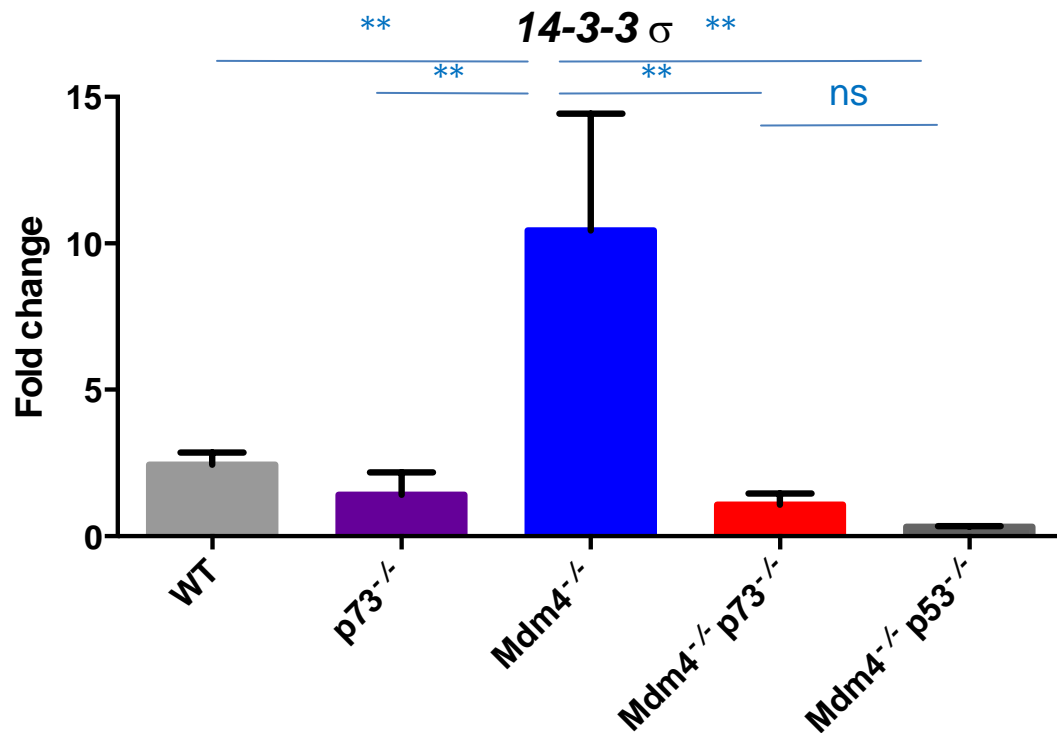


Figure 9. Deletion of *Mmd4* results in the upregulation of *14-3-3σ*, a *p73* and *p53* common target gene, in the embryonic brain. Real-time RT-qPCR was performed for *14-3-3σ* mRNAs in the wild type (n=7), *p73*^{-/-} (n=7), *Mdm4*^{-/-} (n=6), *Mdm4*^{-/-} *p73*^{-/-} (n=7) and *Mdm4*^{-/-} *p53*^{-/-} (n=4) embryonic brains at E14.5. ** p < 0.01, ns p > 0.05 by ANOVA and Newman-Keuls multiple comparisons test. Error bars: SEM.

Perp

Perp is another p53 target gene, which mediates apoptosis (178) in a cell context-dependent manner (179). More specifically, studies on *Perp*-deficient mice have shown that *Perp* exerts its p53-dependent apoptosis in the developing CNS as well as thymocytes; however, it is dispensable for apoptosis in E1A-expressing MEFs (179). Four p53 binding sites have been recognized within the promoter and the first intron of the murine *Perp* gene (180) and it had been suggested that p73 might bind to these sites as well (181).

I therefore examined the effect of *Mdm4* loss on *Perp* expression in the presence and absence of *p53* or *p73*. *Perp* was significantly upregulated in *Mdm4*^{Δ2/Δ2} embryonic brain compared to wild type brains (*p-value* < 0.01), while loss of either *p53* or *p73* abolished its expression (*p-value* < 0.01 and <0.05, respectively) (Figure 10).

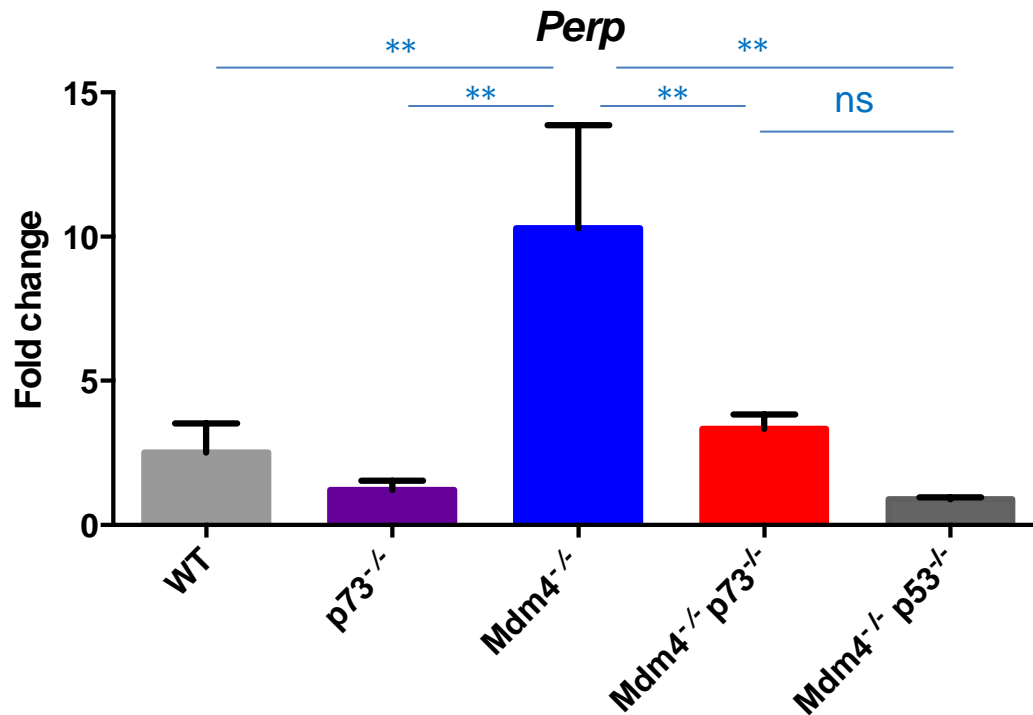


Figure 10. Deletion of *Mmd4* results in the upregulation of *Perp*, a *p73* and *p53* common target gene, in embryonic brain. Real-time RT-qPCR was performed for *Perp* mRNAs in the wild type (n=7), *p73*^{-/-} (n=7), *Mdm4*^{-/-} (n=6), *Mdm4*^{-/-} *p73*^{-/-} (n=7) and *Mdm4*^{-/-} *p53*^{-/-} (n=4) embryonic brains at E14.5. ** p< 0.01, ns p >0.05 by ANOVA and Newman-Keuls multiple comparisons test. Error bars:SEM.

p21

p21 is the well-characterized canonical transcriptional target of p53 (182, 183) and a major mediator of p53-dependant cell cycle arrest. It was also recognized as one of the first p73 target genes in the cells overexpressing wild type *p73*; yet, unlike p53, p73 does not induce *p21* in response to DNA damage (174). Moreover, the level of p21 induced by p53 was three to six times higher than p73 (122).

I also examined the level of *p21* in my model system and observed that *p21* was only upregulated in *Mdm4*-deficient embryonic brain in a p53-dependent manner (*p-value* < 0.01) (Figure 11).

p73 specific target genes

I also examined a number of *p73* specific target genes, which modulate neurogenesis, including *p57* (184, 185), *Jag1* (186), *Jag2* (121, 173, 186), *Hes5* (172, 173) and *Notch2* (173); however, I did not observe any statistically significant increase in expression of these targets as a result of *Mdm4* loss (data not shown).

Combined, these observations highlight the dominance of p53 activity, which results in extensive cell cycle arrest and apoptosis in *Mdm4*-deficient embryonic brain and compromises the other pathways in the developing CNS.

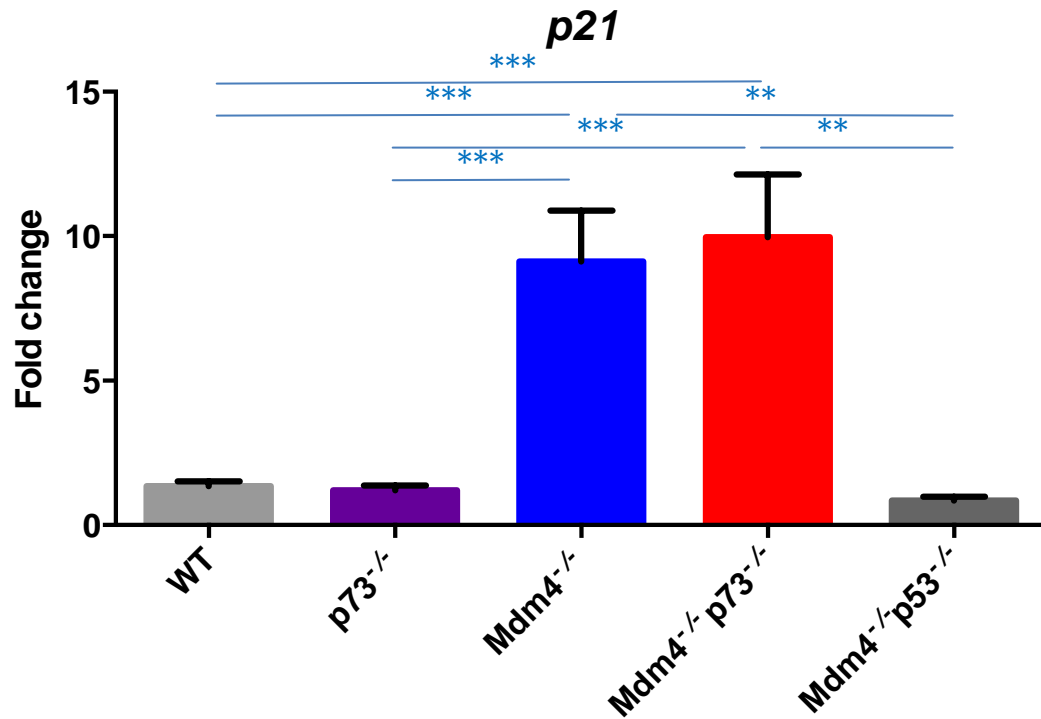


Figure 11. Deletion of *Mmd4* results in the upregulation of *p21* in the embryonic brain in a *p53*-dependent manner. Real-time RT-qPCR was performed for *p21* mRNAs in the wild type (n=7), *p73*^{-/-} (n=7), *Mdm4*^{-/-} (n=6), *Mdm4*^{-/-} *p73*^{-/-} (n=7) and *Mdm4*^{-/-} *p53*^{-/-} (n=4) embryonic brains at E14.5. *** p < 0.001 , ** p < 0.01, *p<0.05, ns p >0.05 by ANOVA and Newman-Keuls multiple comparisons test. Error bars:SEM.

The cooperation of the Mdm4 and p73 during tumorigensis

Given that overexpression of *Mdm4* and loss of *p73* have been implicated in tumor development, I asked whether these two alterations cooperate during tumorigenesis. *Mdm4*^{Tg15} is one of three generated *Mdm4* transgenic mouse lines in our lab, which develop spontaneous tumorigenesis (41). *Mdm4*^{Tg15} MEFs express at least five times higher levels of Mdm4 compared to wild type MEFs and have dampened p53 activity (Figure 12). I crossed *Mdm4*^{Tg15} with *p73*^{+/-} mice and established a cohort of *Mdm4*^{Tg15} *p73*^{+/-} (23 mice), *Mdm4*^{Tg15} (17 mice), *p73*^{+/-} (13 mice) and wild type (5 mice) mice in C57BL/6 background. Since I initially planned to include *Mdm4*^{Tg15} *p73*^{-/-} and *p73*^{-/-} mice to the cohort, I also crossed *Mdm4*^{Tg15} *p73*^{+/-} with *p73*^{+/-} mice. However, no *Mdm4*^{Tg15} *p73*^{-/-} mouse was viable after postnatal day 21 (P21) due to malnutrition and growth retardation. There is also a possibility that C57BL/6 background contributed to the early lethality of these pups. In the tumor study, all mice were sacrificed due to poor health conditions or upon reaching the two-year endpoint of this study.

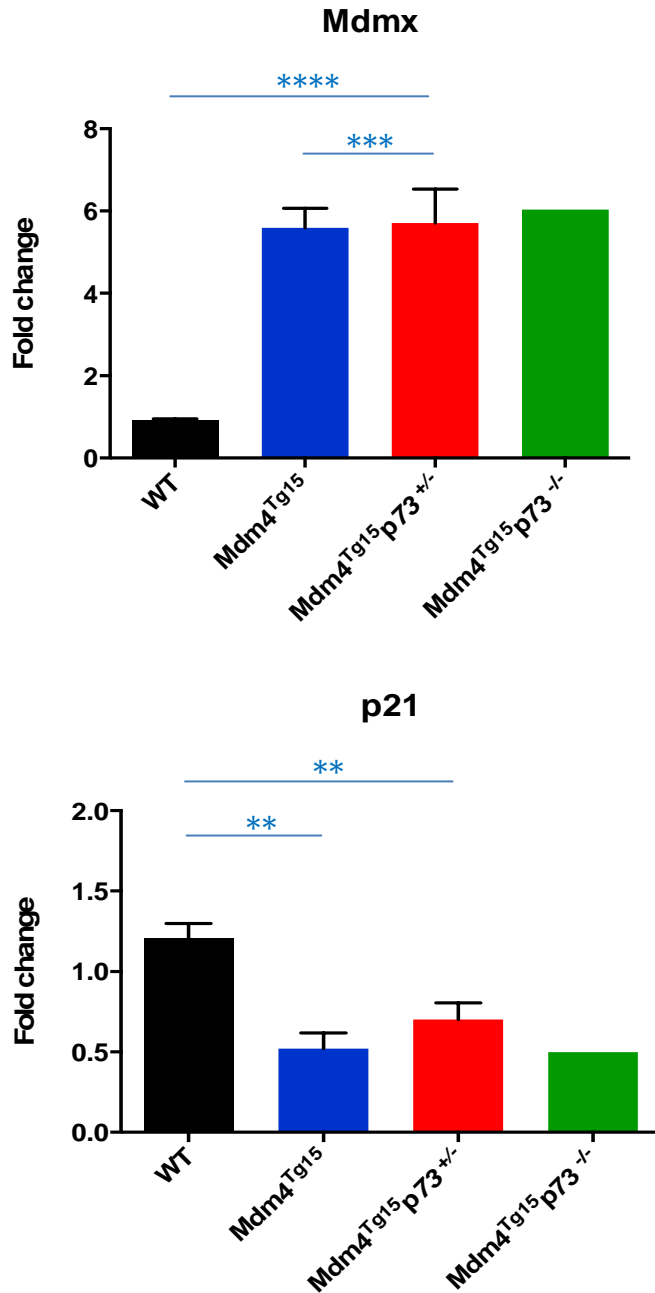


Figure 12. Level of *Mdm4* and *p21* transcripts in MEFs. Real-time RT-qPCR was performed for *Mdm4* and *p21* mRNAs in the wild type (n=6), *Mdm4*^{Tg15} (n=4), *Mdm4*^{Tg15} p73^{+/-} (n=4), *Mdm4*^{Tg15} p73^{-/-} (n=1) MEFs. **** p < 0.0001 , ** p < 0.01, by ANOVA and Newman-Keuls multiple comparisons test. Error bars:SEM.

At two years, 18 of 23 (78.3%) *Mdm4^{Tg15} p73^{+/-}* mice had been sacrificed, compared to 15 of 17 (88.2%) *Mdm4^{Tg15}* and 1 of 13 (7.7%) *p73^{+/-}* mice. Both *Mdm4^{Tg15} p73^{+/-}* and *Mdm4^{Tg15}* mice had significantly shorter life span compared to *p73^{+/-}* and wild type littermates (*p-value* <0.0001) and their median survival was 593 and 665 days, respectively. However, the difference between the survival of *Mdm4^{Tg15} p73^{+/-}* and *Mdm4^{Tg15}* mice was not significant (Figure 13).

I further examined the tumor frequency and spectrum of each group. 78.2% (18 of 23) of *Mdm4^{Tg15} p73^{+/-}* and 82.3% (14 of 17) *Mdm4^{Tg15}* mice develop tumor by two years of age, compared to 23% (3 of 13) of *p73^{+/-}* mice. Moreover, *Mdm4^{Tg15} p73^{+/-}* and *Mdm4^{Tg15}* mice had comparable tumor phenotypes, mainly B-cell lymphoma, histiocytic or dendritic cell sarcoma and brain tumors, whereas *p73^{+/-}* mice developed B-cell lymphoma, histiocytic or dendritic cell sarcoma and angiosarcoma (Figure 14) (Table 8). All tumors were initially diagnosed morphologically and, in case of lymphoma or sarcoma, the diagnoses were further confirmed by immunohistochemistry studies for makers such as CD45R /B220, CD3 and lysozyme. All lymphomas were strongly positive for surface marker CD45R /B220, implying that they were of B-cell origin (Figure 15).

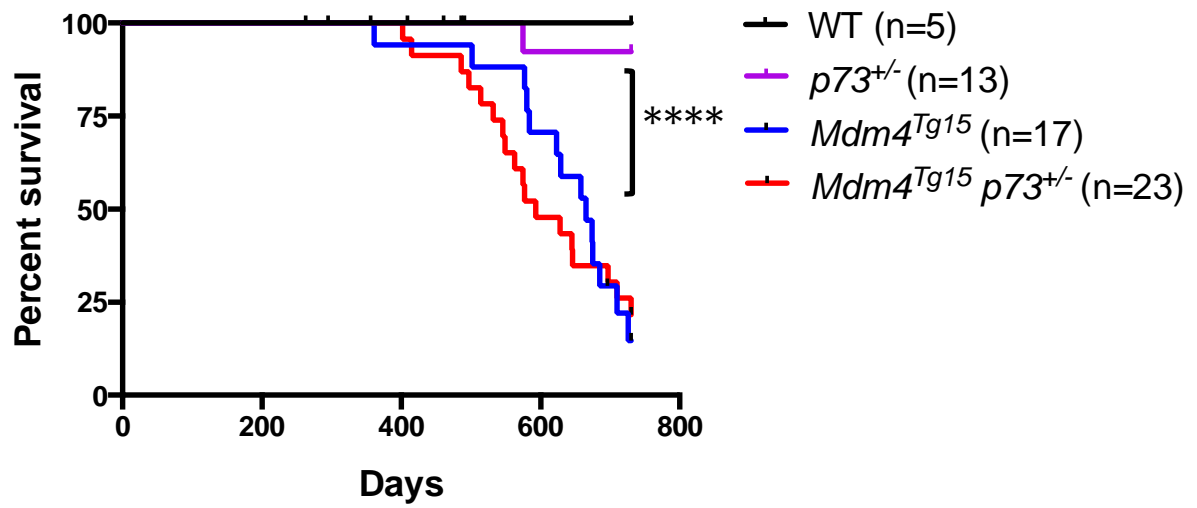


Figure 13. Kaplan-Meier survival curves of $Mdm4^{Tg15} p73^{+/-}$, $Mdm4^{Tg15}$, $p73^{+/-}$ and wild type mice. The number of animals per genotype is indicated.

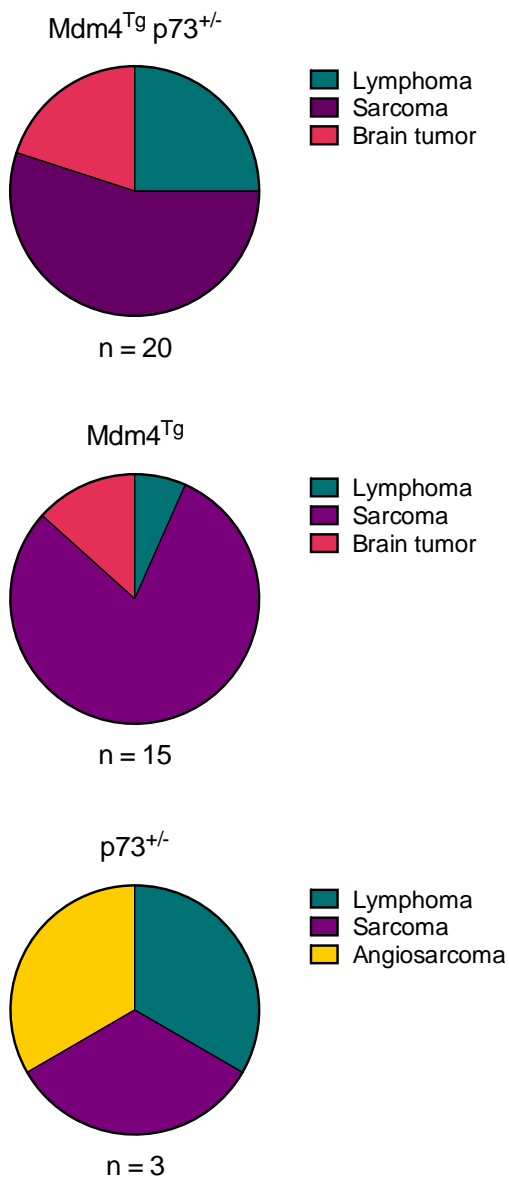


Figure 14. The tumor spectrum of *Mdm4^{Tg15} p73^{+/-}*, *Mdm4^{Tg15}* and *p73^{+/-}* mice. n is the total number of tumors.

Table 8. Spontaneous tumor spectrum of *Mdm4^{Tg15} p73^{+/-}*, *Mdm4^{Tg15}* and *p73^{+/-}* mice

Tumor type	Genotype		
	<i>Mdm4^{Tg15} p73^{+/-}</i> ^a (n=18) [#]	<i>Mdm4^{Tg15}</i> ^b (n=14) [#]	<i>p73^{+/-}</i> ^c (n=3) [#]
Lymphoma	5 (25 %) [§]	1 (6.7 %)	1 (33.3 %)
Sarcoma	11 (55 %) [¶]	12 (80 %) [*]	1 (33.3 %)
Brain tumor	4 (20 %)	2 (13.3 %)	0
Unclassified large cell tumor	4	1	0
Choroid plexus tumor	0	1	0
Angiosarcoma	0	0	1 (33.3 %)
Total number of tumors	20	15	3

^a Among 23 *Mdm4^{Tg15} p73^{+/-}* mice, 18 mice were sacrificed due to moribund condition. Sixteen of them were diagnosed with tumor and 2 mice had two primary tumors. Among 5 mice sacrificed due to 2-year time point, two mice were diagnosed with tumor.

^b Among 17 *Mdm4^{Tg15}* mice, 15 mice were sacrificed due to moribund condition. Fourteen of them were diagnosed with tumor and one mouse had two primary tumors. Between 2 mice sacrificed due to 2-year time point, one mouse was diagnosed with tumor.

^c Among 13 *p73^{+/-}* mice, one mouse was sacrificed due to moribund condition and diagnosed with tumor. Among 12 mice sacrificed due to 2-year time point, two mice were diagnosed with tumor.

[#] n is the number of mice histopathologically diagnosed with tumor.

[§] 4 out of five mice had disseminated lymphoma (infiltration in more than one organ). Two mice including the one with localized lymphoma had multiple primary tumors.

[¶] 6 out of eleven *Mdm4^{Tg15} p73^{+/-}* mice had sarcoma in more than one organ.

^{*} 10 out of twelve *Mdm4^{Tg15}* mice had sarcoma in more than one organ.

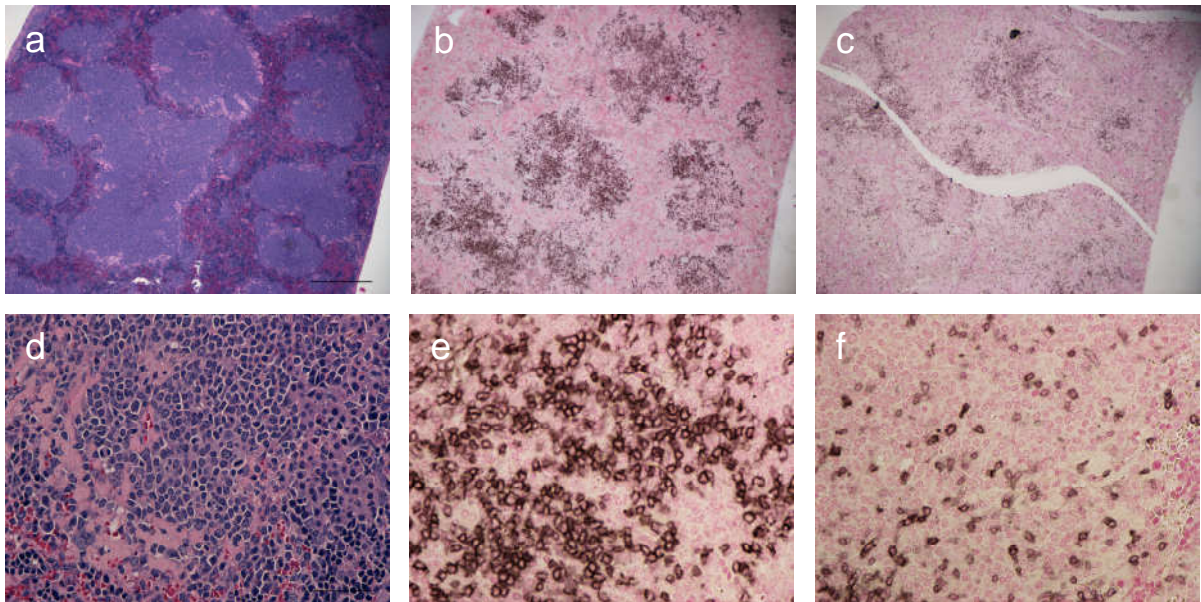


Figure 15. Representative histopathological sections stained with H&E (a, d) and subjected to immunohistochemistry for B-cell marker (CD45R /B220) (b, e) and T-cell marker (CD3) (c, f) of the spleen of an *Mdm4*^{Tg15} *p73*^{+/-} mouse with B-cell lymphoma. Magnification, x4 for a-c and x40 for d-f.

Similar to other tumor suppressor genes, *p73* undergoes LOH during tumorigenesis. To determine whether the tumors from *Mdm4^{Tg15} p73^{+/-}* mice lose the wild type allele, I extracted DNA from tumor tissue or nodules of lymphoma in the spleen or liver and analyzed by PCR. Interestingly, all the tumors from *Mdm4^{Tg15} p73^{+/-}* mice retained the wild type *p73* allele (Figure 16), whereas at least 40% of *p73^{+/-}* tumors has been reported to undergo LOH (166), suggesting that overexpressed Mdm4 might be inhibiting the *p73* wild type allele in *Mdm4^{Tg15} p73^{+/-}* tumors.

Intriguingly, four *Mdm4^{Tg15} p73^{+/-}* mice had unclassified large cell brain tumors compared to only one *Mdm4^{Tg15}* mouse (20% of *Mdm4^{Tg15} p73^{+/-}* mice compared to 6.6% of *Mdm4^{Tg15}* mice). Given that a subset of brain tumors has amplification of *Mdm4* and/or homozygous deletion of *p73* (98) and that molecular findings in this study suggest the inhibitory effect of Mdm4 on p73 in mouse embryonic brain, the observation of higher number of unclassified large cell brain tumors in *Mdm4^{Tg15} p73^{+/-}* mice compared to *Mdm4^{Tg15}* mice also supports a potential Mdm4-p73 cooperation.

Moreover, five *Mdm4^{Tg15} p73^{+/-}* mice had B-cell lymphoma compared to only one *Mdm4^{Tg15}* mouse (21.7% of *Mdm4^{Tg15} p73^{+/-}* mice compared to 5.8% of *Mdm4^{Tg15}* mice), and each lymphoma was highly disseminated, had another primary tumor such as sarcoma, or had several disseminated foci of lymphoma in lymphoid and nonlymphoid organs as well as multiple primary tumors (Figure 17-20). On the other hand, lymphoma in the *Mdm4^{Tg15}* mouse was localized in only one lymphoid organ, the mesenteric lymph node. Since several studies have suggested a role of p73 in lymphomagenesis (146, 147, 157), increased lymphomagenesis in *Mdm4^{Tg15}*

p73^{+/-} mice indicates a possible cooperation between Mdm4 and p73. Yet, these differences were not statistically significant compared to *Mdm4*^{Tg15} mice, perhaps due to a small sample size.

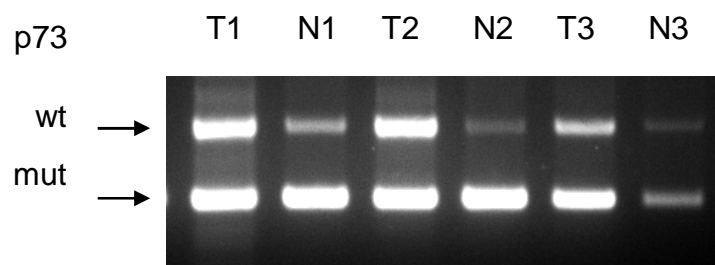


Figure 16. Representative examples of LOH analysis at *p73* loci by genomic PCR of tumor (T) and normal (N) tissues from *Mdm4*^{Tg15} *p73*^{+/-} mice. All tumors retained wild type allele of *p73*.

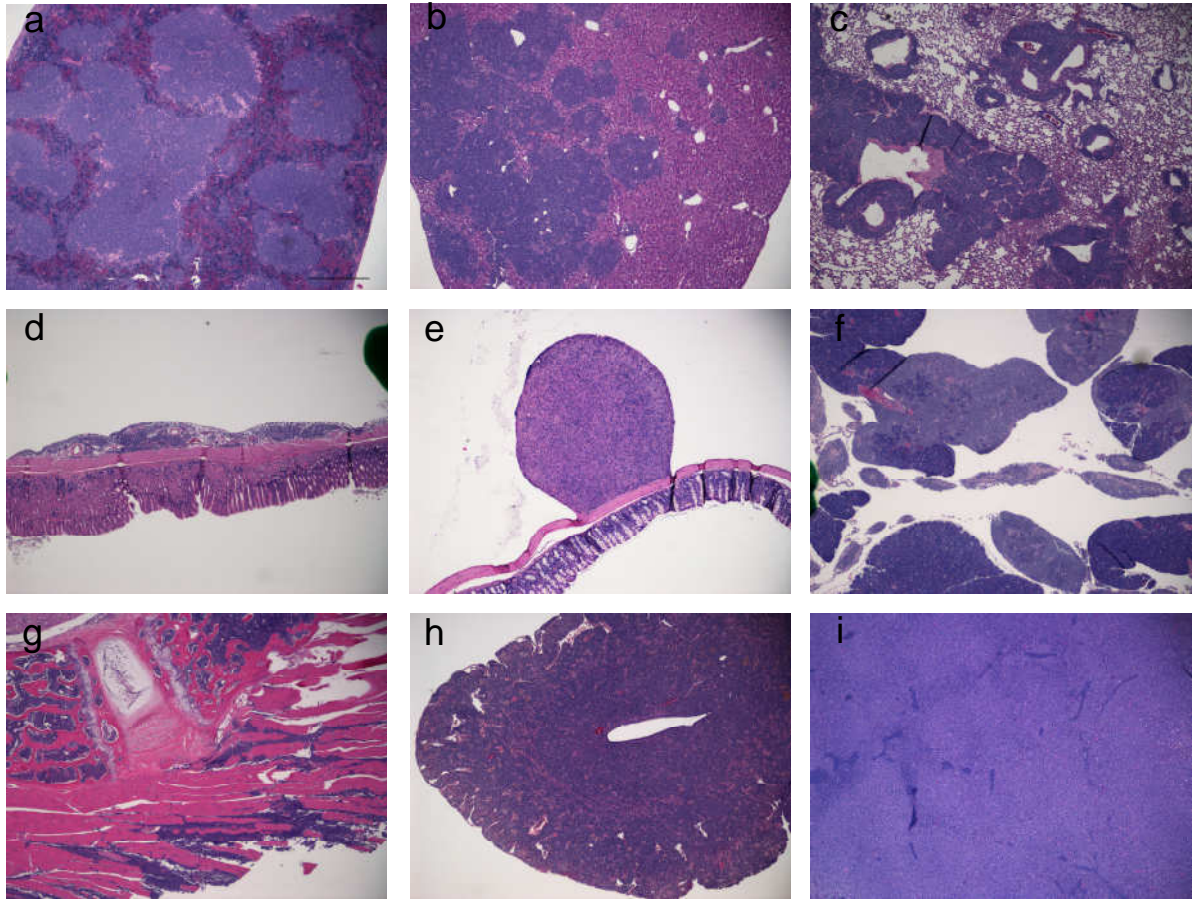


Figure 17. A representative histopathological examination (H&E staining) of a *Mdm4*^{Tg15} *p73*^{+/-} mouse with highly disseminated lymphoma and multiple primary tumors. Lymphoma is disseminated in spleen (a), liver (b), lung (c), serosa of duodenum (d), pancreas (f), paraspinal skeletal muscles (g), uterus (h) and lymph node (the architecture of lymph node is replaced by lymphoma cells) (i). The second primary tumor is sarcoma in colon (e). Magnification, $\times 4$.

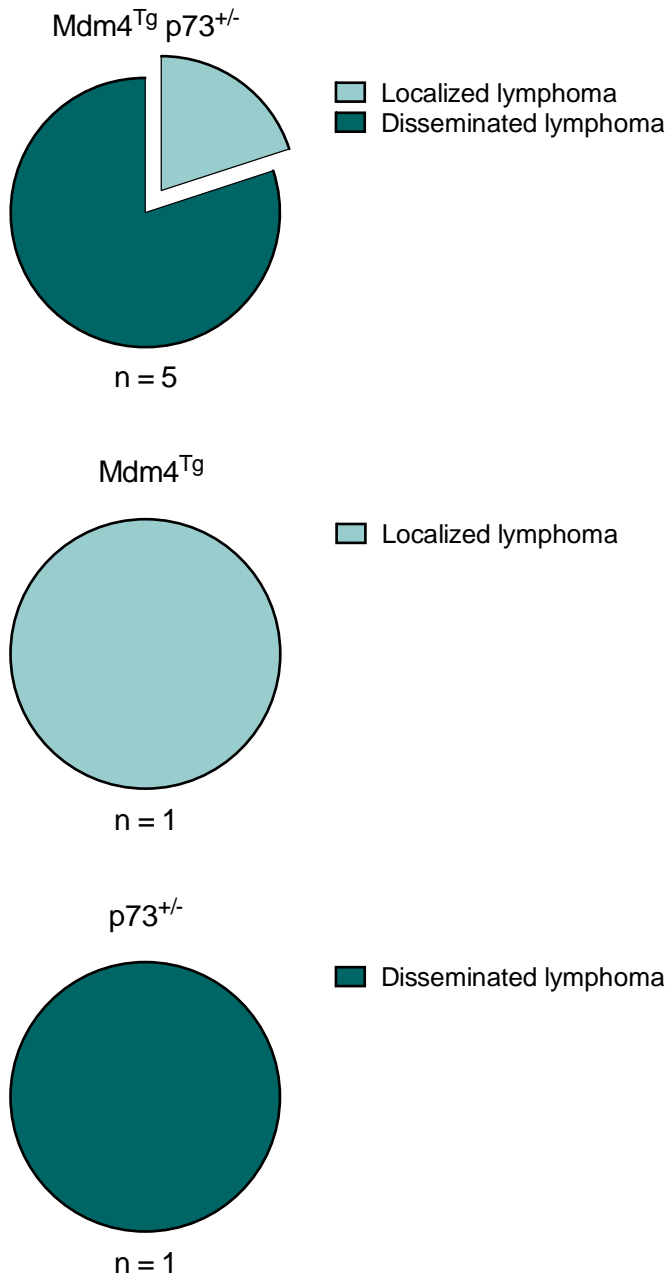


Figure 18. The incidence of dissemination of lymphoma in $Mdm4^{Tg15} p73^{+/-}$, $Mdm4^{Tg15}$ and $p73^{+/-}$ mice. n is the number of mice with lymphoma.

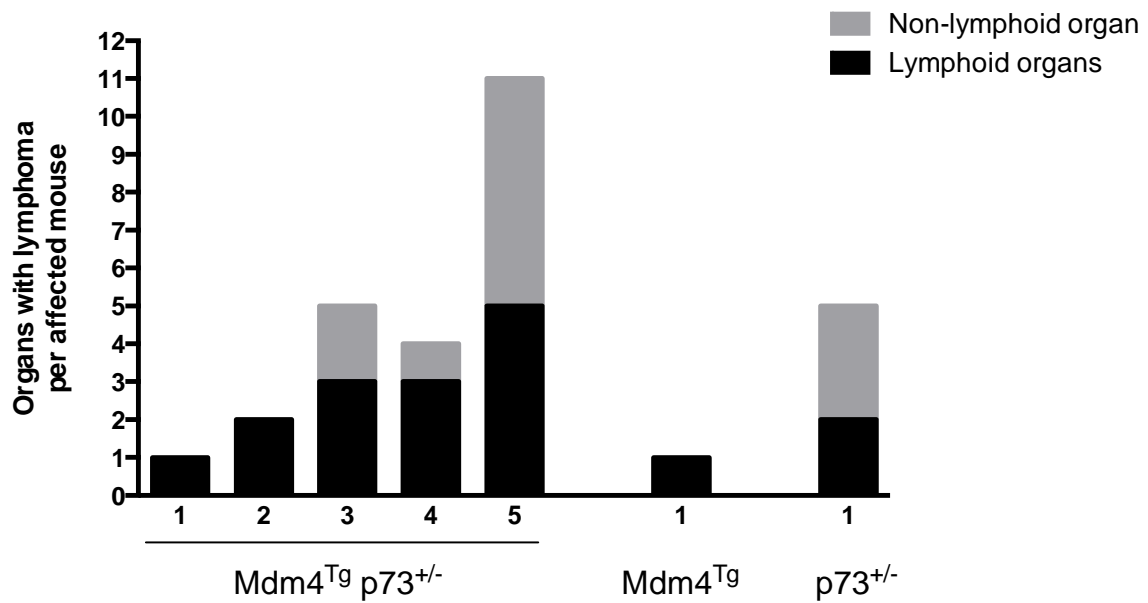


Figure 19. The pattern of dissemination of lymphoma to various organs and number of lymphoid and non-lymphoid organs infiltrated with lymphoma in *Mdm4^{Tg15} p73^{+/-}*, *Mdm4^{Tg15}* and *p73^{+/-}* mice.

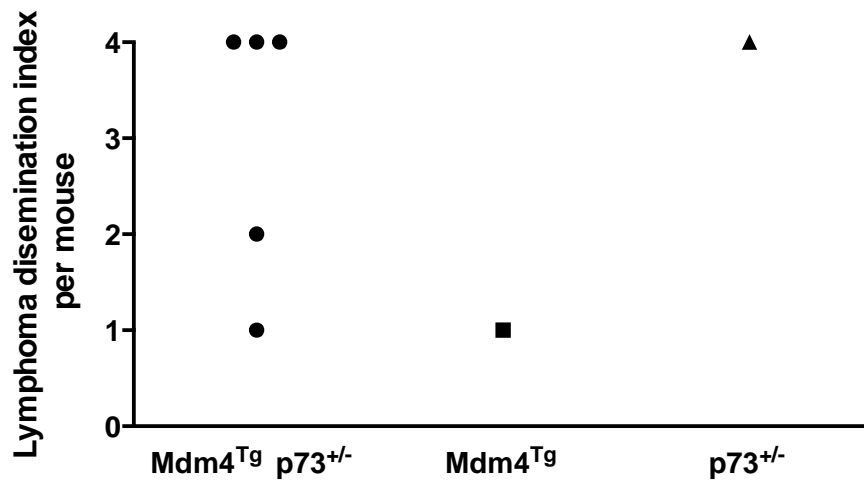


Figure 20. The lymphoma severity index in $Mdm4^{Tg15} p73^{+/-}$ (n=5), $Mdm4^{Tg15}$ (n=1) and $p73^{+/-}$ (n=1) mice. Each mouse with lymphoma is depicted by a dot.

Lymphoma Dissemination Index: 0: No lymphoma, 1: Lymphoma in one or more LNs in one LN groups, thymus or spleen (lymphoid organs), 2: Lymphoma in 2 or more LN groups either above or below diaphragm, 3: Lymphoma in 2 or more LN groups above and below diaphragm, 4: Lymphoma in non-lymphoid tissues

CHAPTER 3. DISCUSSION AND FUTURE DIRECTIONS

The observations that Mdm4 is overexpressed in many tumors and most tumors lack *p73* mutations, suggest a mechanism for inactivation of *p73* in human cancer. Despite the biochemical and *in vitro* studies on the physical binding of Mdm4 and *p73*, thus far no studies have examined the biological significance of this interaction. In my thesis work, using the complicated genetic mouse models, I provide the first comprehensive *in vivo* characterization of the Mdm4 and *p73* interaction during development and tumorigenesis.

My results reveal that loss of *p73* does not rescue *Mdm4*-deficient early embryonic lethality, indicating that the unrestricted p53 activity, in absence of Mdm4 inhibition, leads the phenotype. Interestingly, my data show that Mdm4 might inhibit the *p73* transcriptional activity in mediating cell cycle arrest and apoptosis during brain development and there is a possibility that, in the absence of *Mdm4*, *p73* contributes to the vigorous senescent and apoptotic phenotype of p53 in embryonic brain. However, deletion of *p73* does not rescue the *Mdm4*-null porencephaly phenotype and this phenotype is also mainly under the influence of enhanced p53 activity. Such observation underscores that despite the well-established role of *p73* in neurogenesis, the Mdm4-*p73* axis is not as significant as the Mdm4-p53 pathway during the CNS development.

My tumor study also suggests a possible cooperation between Mdm4 and *p73* during tumorigenesis, as evidenced by increased incidence of lymphoma and brain tumor in *Mdm4*^{Tg15} *p73*^{+/-} mice compared to *Mdm4*^{Tg15} mice. Nonetheless, the differences in survival curve and tumor spectrum are not statistically significant,

implying that the dampened p53 activity due to high levels of Mdm4 is the major driver of spontaneous tumorigenesis in these mice and compromises the potential Mdm4-p73 cooperation.

The Mdm4-p73 interaction during development

Despite the strong *in vitro* evidence of the Mdm4-p73 binding in the mammalian cells and the higher affinity of Mdm4 for p73 rather than p53 in biochemical studies, my *in vivo* studies suggest that, in the presence of enhanced p53 activity, such interaction does not have biological importance in embryogenesis. However, to ask this question in a more relevant biological system and to avoid the strong p53 effect in the whole embryo, I chose to examine the consequences of the Mmd4-p73 binding in the developing CNS.

I found that Mdm4 inhibits the p73-mediated cell cycle arrest and apoptosis in embryonic brain at E14.5, as evidenced by regulation of *14-3-3 σ* and *Perp*, indicating that p73 contributes to *Mdm4*-deficiency brain tissue loss. However, the effect of unrestricted p53 and, subsequently, the activation of plethora of target genes, are much more stronger than the effect of p73 and dominates the *Mdm4* ^{$\Delta 2/\Delta 2$} *p73* ^{$-/-$} brain phenotype. Another explanation for the dominant phenotype of p53 is its expression pattern compared to p73 during CNS development. At E10.5, the expression of p53 is ubiquitous in mouse brain (187) while p73 is expressed sparsely and mostly in telencephalon (141). At later embryonic stage, p53 expression is more heterogeneous with high levels of p53 mRNA in the ventricular zone neuroepithelial progenitors, telencephalon and mesencephalon, and low levels

in the cortical plate (187), whereas p73 is highly expressed in cortical hem, a structure close to hippocampus, and more specifically in CR cells (141). Thus, it seems reasonable to postulate that upon deletion of *Mdm4*, both p53 and p73 have increased activity as evidenced by the upregulation of their target genes, yet each affects distinct parts of embryonic brain. Consequently, p53 with expression in more regions of brain dictates the *Mdm4*^{Δ2/Δ2} p73^{-/-} brain phenotype. Other reason could be the late formation of hippocampus during embryogenesis. Although hippocampal molecular differentiation begins at E10.5 to E12.5, the earliest apparent hippocampal field differentiation is at E15.5. Thus, the time point of my study at E14.5 is relatively early to observe the effect of dysregulated p73 activity in hippocampal formation.

The best way to circumvent enhanced p53 activity due to absence of *Mdm4* is deleting p53 concomitantly. It is well known in the field that *Mdm4*^{Δ2/Δ2} p53^{-/-} mice are viable with the phenotype and life span of p53^{-/-} mice. However, the detailed assessment of brain anatomy, especially with respect to the regions of the brain wherein p73 has roles, has not been studied. For instance, whether *Mdm4*^{Δ2/Δ2} p53^{-/-} mice compared to *Mdm4*^{Δ2/Δ2} p53^{-/-} p73^{-/-} mice have hippocampal defect or cortical malformation due to dysregulated p73 activity has not been examined. The *Mdm4*^{Δ2/Δ2} p53^{-/-} p73^{-/-} mice are viable, however, they die before weaning (based on my observation, they die approximately at P10). Thus, the best time point for such analyses is early postnatal days when the classic morphology of hippocampus is evident (188).

Since p73^{-/-} mice depict the most severe phenotype in brain compared to *TAp73*^{-/-} and *ΔNp73*^{-/-} mice, it was the best genetic model to test our hypothesis.

However, given that N-terminal of Mdm4 binds to TA domain of p73 (167) and that TAp73 is more abundant than Δ Np73 in neural stem cells (172), most probably Mdm4 interacts with TAp73 during CNS development. My observation that cell cycle arrest and apoptosis is the predominant phenotype upon deletion of Mdm4 in brain and that TAp73 is the isoform with apoptotic and growth arrest properties (132, 134) also suggest that TAp73 is the major isoform in the Mdm4-p73 interaction. To test this notion, it would be interesting to perform rescue study in the brain using *TAp73^{+/-}* mice, instead of *p73^{+/-}* mice. In this setting, in the absence of *TAp73*, Δ Np73 is supposed to exert an inhibitory effect on p53-mediated apoptosis (137) and the experiment might be less confounded by the high p53 activity. Moreover, this experiment might shed light to the interplay of *TAp73* with Δ Np73 and the importance of the ratio of these isoforms during CNS development, the concept that have been investigated in cultured neurospheres, but not *in vivo* and during embryogenesis.

Clinical implication

Congenital porencephaly is a rare anomaly in the neonate's brain due to aberrant development of CNS (189). It is characterized as a cavity in cerebral hemispheres and causes severe seizures and mental retardation in patients (189). It is mostly sporadic and have been related to trauma and ischemic injury at mid-gestation (190), but the observations of familial porencephaly suggested the genetics could also be involved in a subset of patients (191). Thus far, dysregulation of the Mdm4-p53 pathway have not been studied directly. However, *COL4A1* is among the limited mutations that have been found to be associated with this

anomaly (192-194). Surprisingly, this gene is a p53 target gene (195), which indicates that p53 pathway could be activated in porencephaly. Interestingly, hippocampal atrophy has been reported in 95% of cases with congenital porencephaly (196). Such striking coexistence implies a common mechanism underlying both pathologies; however, it remained to be elucidated.

Despite the prominent role of p73 during CNS development and in the maintenance of neural stem cells, there is still no report on the role of this gene in the pathogenesis of congenital brain defects. Together, the observation of porencephaly in *Mdm4*-null mice, the *Mdm4*-p73 interaction in developing CNS and the prominent role of p73 in the formation of hippocampus, suggest that it is worth examining the genetic alteration of *Mdm4* and *p73* as well as their protein-protein interaction in the subset of patients with porencephaly and hippocampal atrophy.

The *Mdm4*-p73 cooperation during tumorigenesis

Some neoplasms such as lymphoma and brain tumor have been shown to harbor both *Mdm4* overexpression and/or *p73* loss. To address whether these two alterations occur within a tumor and, more specifically, if they happen in one tumor cell and consequently cooperate in tumor development, I used genetic mouse model with high expression of *Mdm4* and decreased *p73* gene dosage.

My tumor study revealed that either *Mdm4* overexpression or *p73* heterozygosity is tumorigenic, which is consistent with previous studies (41, 155). *Mdm4*^{Tg15} *p73*^{+/-} mice also developed spontaneous tumors, and, interestingly, they

had shorter survival, higher frequencies of lymphoma and brain tumor, as well as strikingly highly disseminated lymphoma compared to each individual alteration.

Notably, *Mdm4*^{Tg15} mice in my cohort had longer survival and developed tumor with more latency compared to our published *Mdm4*^{Tg15} cohort study. Such difference between two cohorts of one transgenic mouse line might result from the altered pattern of transgene expression over serial breeding and across generations, otherwise the genetic background of both was C57BL/6 and they both had the same expression level of *Mdm4* in MEFs (4-5 times higher than wild type MEFs) (41). On the other hand, *p73*^{+/-} mice in my cohort had prolonged survival along with a very low frequency of tumorigenesis. Such discrepancy between *p73*^{+/-} mice in my cohort and published study could be due to mouse genetic background, the factor that has already been shown to affect the rate and spectrum of tumor development in other mouse models such as *p53*^{-/-} mice (197, 198). The *p73*^{+/-} mice in my cohort were in >95% C57BL/6, whereas in the previous study these mice had mixed genetic background of C57BL/6 and 129/SvJae (155).

Subsequently, such alterations in transgene expression and mouse genetic background also affected the survival and tumor spectrum of *Mdm4*^{Tg15} *p73*^{+/-} mice and perhaps buffered the tumor phenotype. However, in essence, such effects made the interpretation of *Mdm4*^{Tg15} *p73*^{+/-} tumor phenotype easier: *p73* heterozygosity in C57BL/6 background barely develops tumors, whereas *Mdm4* overexpression develops tumor, yet the rates of lymphoma and brain tumor are very low. However, the combination of both alterations results in more aggressive phenotype, higher incidence of severely disseminated lymphoma and higher incidence of brain tumor.

The presence of wild type *p73* allele, as evidenced by lack of LOH, in the *Mdm4*^{Tg15} *p73*^{+/-} tumors also indicates that either Mdm4 overexpression dampens the tumor suppressor activity of remaining *p73* allele or loss of only one allele of *p73* in the context of high levels of Mdm4 does suffice for tumor development due to their collaboration through cancer signaling pathway(s).

Since the tumor survival and tumor spectrum of *Mdm4*^{Tg15} *p73*^{+/-} mice is highly suggestive of cooperation, yet not statistically significant, there is a possibility that higher level of Mdm4 would be required in order to robustly show this cooperation. For that, using a homozygous *Mdm4* transgene and comparing *Mdm4*^{Tg15/Tg15} *p73*^{+/-} to *Mdm4*^{Tg15/Tg15} and *p73*^{+/-} mice could be more revealing.

Moreover, observing another tumor type in *Mdm4*^{Tg15} *p73*^{+/-} mice, which is known, based on data from human cancer, to have altered *Mdm4* and *p73* would also be supportive. For instance, similar to lymphoma and brain tumor, the role of Mdm4 overexpression and loss of *p73* have been well studied in breast tumors. Since C57BL/6 mice are resistant to mammary tumor development (199), unfortunately there was no chance for such observation in my model system. On the other hand, Balb/c mice are prone to this type of tumor (199) and, interestingly, *Mdm4* transgenic mouse in the Balb/c genetic background develop mammary tumor (data from our lab). However, the *p73*^{+/-} tumorigenicity in this genetic background has not yet been reported. It would be interesting to observe the phenotype of *p73* heterozygosity and, further, examine *Mdm4*^{Tg15} *p73*^{+/-} mice in Balb/c background for mammary tumor formation and enhanced metastatic potential.

Furthermore, given that TAp73 is the isoform with tumor suppressor activity, it would be interesting to establish a cohort of *Mdm4*^{Tg15} *TAp73*^{+/-} and *Mdm4*^{Tg15} *TAp73*^{-/-} mice to decipher this cooperation in an isoform-specific manner and also to understand the role of Mdm4 in regulating the ratio of both *TAp73* and $\Delta Np73$ isoforms during tumorigenesis.

One potential mechanism by which this cooperation contributes to tumorigenesis could be genomic instability. Elevated levels of Mdm4 increase chromosome and chromatid breaks and delay DNA repair in a p53-independent manner (87). In addition, loss of *p73* is also associated with genome instability (126, 128). Thus, it is worthy to investigate whether *Mdm4*^{Tg15} *p73*^{+/-} and *Mdm4*^{Tg15} *p73*^{-/-} mice, compared to *Mdm4*^{Tg15}, have higher levels of chromosome defects in tumor-prone tissues, such as spleen and more specifically B-cells, at the pre-malignant stage, for example at 6-9 months old.

Another possible mechanism through which Mdm4 overexpression and p73 heterozygosity could collectively enhance tumorigenicity might be E2F-1 modulation. The transcription factor E2F-1 mediates apoptosis and cell cycle arrest (200). *p73* has been identified as one of E2F-1 target genes and, in the absence of *p53*, E2F-1 mediates apoptosis through upregulation of *p73* (201). Given that Mdm4 associates with E2F-1 and inhibits its transcriptional activity (89), it is reasonable to postulate that in *Mdm4*^{Tg15} *p73*^{+/-} tumors, along with dampened p53-mediated cell death, p73-dependent apoptosis is decreased due to E2F-1 inhibition. Another support for this notion is the role of E2F-1 in lymphoma and brain tumors. It has been reported that the low level of E2F-1 is associated with poor prognosis in patients with B-cell

lymphoma (202) and that E2F-1 has been considered as one the therapeutic tools in cancer gene therapy for brain tumors (203, 204).

Clinical implication

A thorough investigation in The Cancer Genome Atlas (TCGA) on lymphoma, brain tumor, and probably breast cancer, would shed light on the coexistence of *MDM4* and *p73* alterations in tumors and, hopefully, might identify the other alterations as a mediator or consequence of their cooperation. The collection of such alterations could be used as a prognostic factor or to classify a subset of patients who benefit from targeted therapy.

CHAPTER 4. MATERIALS AND METHODS

Mice

Mdm4 heterozygous knockout mouse was generated in our lab (80). *Mdm4* ^{$\Delta 2$} allele is the null allele of *Mdm4*, which is generated by deletion of exon 2 in *Mdm4*^{*fx/+*} mice, and no Mdm4 protein was detected by western blot in *Mdm4* ^{$\Delta 2/\Delta 2$} *p53*^{*-/-*} MEFs (80).

Mdm4^{*+/\Delta 2*} mice (80), *p73*^{*+/-*} mice (155) (from Dr. Elsa Flores, MD Anderson Cancer Center, Houston, TX) and *p53*^{*+/-*} (205) mice on a mixed background (129/SvJae and C57BL/6) were crossed to generate breeders with genotype of interest for the rescue (Figure 21) and gene dosage study on the whole embryo (Figure 22).

Mdm4^{*+/fx*} mice (80), *p73*^{*+/-*} mice (155), and *Nes-cre* transgenic mice (170) (from Jackson laboratory) on C57BL/6 background were crossed to generate breeders with genotype of interest for the rescue study on developing CNS (Figure 23).

The day a plug was detected in females was assigned as E0.5 and the day of birth is defined as P0. In the first experiment of gene dosage study, mice were genotyped at P7; however, from P0 to P7 they were checked and counted daily.

Mdm4^{*Tg15*} mice (41) and *p73*^{*+/-*} mice (155), both on over 95% C57BL/6 background, were crossed to generate the cohort for tumor study (figure 24). Mice were sacrificed upon moribundity or reaching 2-year time point and necropsies were performed.

$Mdm4^{+/\Delta 2} \times p73^{+/-}$



$Mdm4^{+/\Delta 2} p73^{+/-} \times Mdm4^{+/\Delta 2} p73^{+/-}$



At specific time point: Sacrifice pregnant female

Figure 21. Genetic scheme for the rescue study

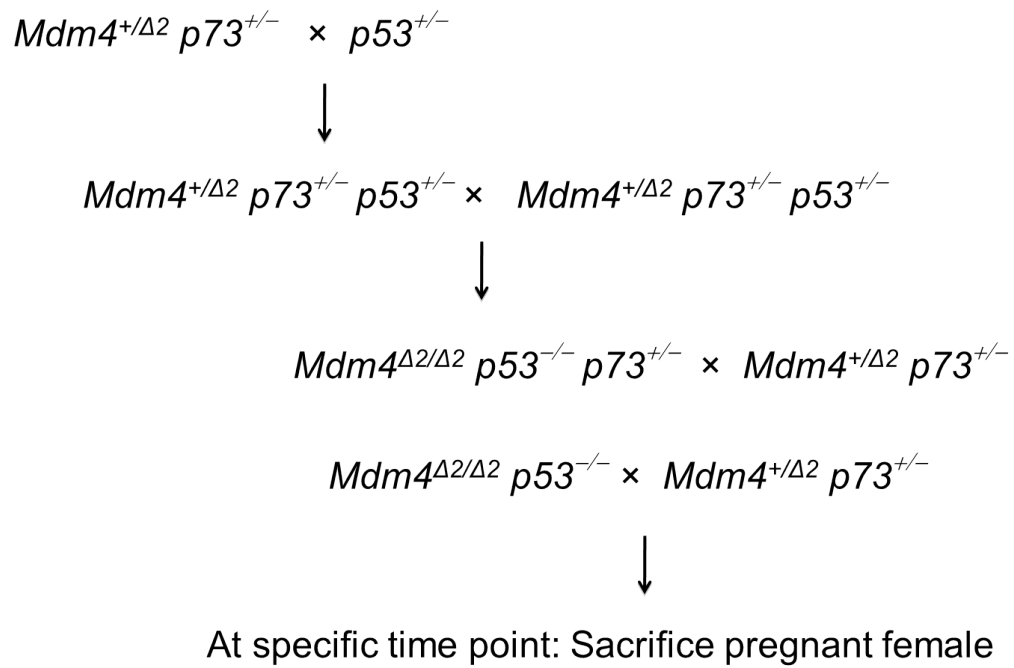


Figure 22. Genetic scheme for the gene dosage study

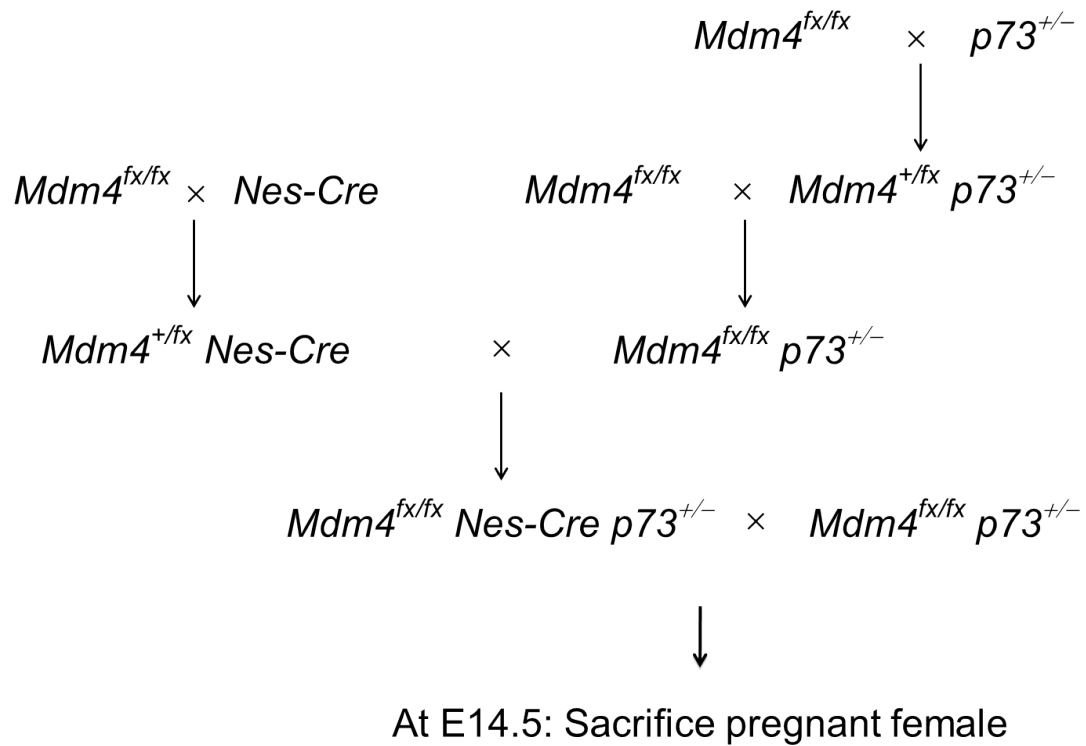


Figure 23. Genetic scheme for the rescue study in developing CNS

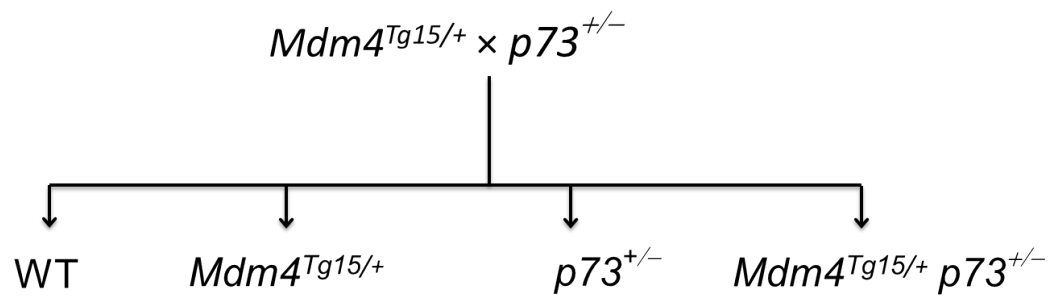


Figure 24. Genetic scheme for establishing the cohort for tumor study

All mice were maintained in the mouse facility at the University of Texas M.D. Anderson Cancer Center in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

In development studies, DNA was extracted from tail or yolk sac, depending on the developmental stage and size of embryo. In tumor study, tail snips were used for DNA extraction. PCR was performed using appropriate PCR primers (Table 9) and based on the available protocols in our laboratory.

Table 9. List of PCR genotyping primers

Gene	primer (5' → 3')	
<i>Mdm4^{fx}</i>	F	ctgggccgaggtggaatgtgatgt
	R	ggtgtcctgaacttgctgttagaa
<i>Mdm4^{Δ2}</i>	F	ttccagagacatgttattcac
	R	tagaatctggaattacagacag
<i>p73</i>	A	ggccatgcctgtctacaaagaa
	B	ccttctacacggatgaggtg
	C	gaaagcgaaggagcaaagctg
<i>p53</i>	A	agcgtggtgtaccttatgagc
	B	ggatggtggtatactcagagcc
	C	tcc tcg tgc ttt acg gta tc
<i>Nes-cre</i>	F	ccaacagttcatgaggattcca
	R	ttggtgtacggtcagtaaattgga
<i>Mdm4^{Tg}</i>	F	agggcgggggtcggcttctgg
	R	tcccaaaagatctccaccacagta

Histopathology and Immunohistochemistry

In development studies, whole embryos were fixed in 4% paraformaldehyde and paraffin embedded for sagittal sectioning. In tumor studies, upon euthanasia of moribund mice, a complete necropsy was performed and all collected organs were fixed in 10% formalin for 48 hours. Fixed samples were embedded in paraffin for sectioning. Histological preparation of embryos and tissue samples as well as hematoxylin and eosin (H&E) staining were performed by the Department of Veterinary Medicine at the University of Texas M.D. Anderson Cancer Center. Dr. M James You kindly recommended the required immunohistochemical analyses and performed pathology on samples in tumor study.

Immunohistochemical staining was performed on paraffin-embedded tissue sections as previously described (84, 166). Antibodies used for immunohistochemistry were: Ki67, 1:100 (ab16667, Abcam, Cambridge, MA, USA); caspase 3, 1:200 (9661S, Cell Signaling Technology, Danvers, MA, USA); CD45R/B220, 1:50 (Clone RA3-6B2 (RUO), BD Pharmingen, San Diego, CA, USA); CD3, 1:100 (ab5690, Abcam, Cambridge, MA, USA; Lysozyme, 1:1000 (NBP1-95509, Novus, Cambridge, UK).

Subsequently, stained sections were detected with Vectastain Elite ABC Reagent and Vector DAB substrate (Vector Laboratories, Burlingame, CA, USA) and counterstained with nuclear fast red (Vector Laboratories).

LOH analyses

A small section of tumor or tumoral foci were dissected and frozen for tumor DNA extraction. PCR was performed for detecting wild type and null alleles of *p73*.

Real-Time qRT-PCR

Total RNAs were isolated from MEFs or embryonic brain at E14.5 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase (Roche Life Science, Indianapolis, IN, USA), and then reverse transcribed by using First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA) following manufacturer's instructions. Quantitative real-time PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems) according to the manufacturer's instructions (Bio-Rad, Valencia, CA, USA). Expression was normalized to the *Rplp0* levels. Primer sequences are in Table 10.

Table 10. List of primers used for Real-Time PCR

Gene	primer (5' → 3')	
<i>p21</i>	F	CAGGCACCATGTCCAATCCT
	R	GAGACAACGGCACACTTTGCT
<i>Mdm4</i>	F	TGAACATTTTACCTTGCGCACCTG
	R	CAACATCTGACAGTGCTTGCAGGA
<i>Perp</i>	F	GCTGCAGCCACGCTTTTC
	R	GGCGAAGAACGAGAGAATGAA
<i>14-3-3σ</i>	F	TGGCCACTGGCGATGAC
	R	GGCTGACCGGGCAGAAT
<i>p57</i>	F	GGCATTGTGGATGAGTGTTG
	R	TCTCCTTTGCAGCTTCGTTT
<i>Hes5</i>	F	AGTCCCAAGGAGAAAAACCGA
	R	GCTGTGTTTCAGGTAGCTGAC
<i>Notch2</i>	F	ATGTGGACGAGTGTCTGTTGC
	R	GGAAGCATAGGCACAGTCATC
<i>Jag1</i>	F	CAAAGTGTGCCTCAAGGAGTATCAG
	R	TCCACCAGCAAAGTGTAGGACCTC
<i>Jag2</i>	F	CAGTGCAAAAACCTTACACCGCCGC
	R	GGAGCAGCTGGGGTCTTTGGTG
<i>Rplp0</i>	F	CCCTGAAGTGCTCGACATCA
	R	TGCGGACACCCTCCAGAA

Statistical Analyses

All statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA) and a p-value of <0.05 was considered statistically significant. The difference between observed and expected frequencies of embryos was determined by chi-square test. The multiple comparisons test for gene expression analyses of embryonic brain was performed by ANOVA using Newman-Keuls posttest. The difference between survival curves was determined by using Log-rank (Mantel-Cox) test and the difference of the tumor spectrum of each group of mice was analyzed by Fisher's exact test.

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