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p53 Activity Dominates that of p73 upon *Mdm4* Loss in Development and Tumorigenesis

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Abstract

Mdm4 negatively regulates the p53 tumor suppressor. *Mdm4* loss in mice leads to an embryonic lethal phenotype that is p53-dependent. Biochemical studies indicate that Mdm4 also binds p73, a member of the p53 family, with higher affinity than p53. In this study, the significance of the Mdm4 and p73 interaction *in vivo* during embryogenesis and tumorigenesis was examined. The data revealed that p73 loss does not rescue either the early *Mdm4*-deficient embryonic lethality or the runt phenotype of *Mdm4*^{2/2} p53^{+/-} embryos. Furthermore, studies in the developing central nervous system (CNS) wherein both genes have prominent roles indicated that loss of p73 also did not rescue the *Mdm4*-null brain phenotype as does p53 loss. This p53 dependency occurred despite evidence for p73-specific transcriptional activity. In tumor studies, the combination of *Mdm4* overexpression and p73 loss did not alter survival of mice or the tumor spectrum as compared to *Mdm4* overexpression alone. In summary, these data demonstrate that the Mdm4-p73 axis cannot override the dominant role of p53 in development and tumorigenesis.

Implications—Genetic characterization of the Mdm4 and p73 interaction during development and tumorigenesis suggests new insight into the role of p53 family members, which may influence treatment options for patients.

Introduction

The tumor suppressing function of p53 is dampened in the majority of human cancers (1). This alteration can be through deletion or mutation of the *TP53* gene itself, or its functional inactivation via upregulation of its negative regulators, MDM2 and MDM4 (2). Several studies and multiple mouse models have elucidated the importance of Mdm2 and Mdm4 as *bona fide* regulators of p53 in embryonic development, normal physiology, DNA damage, and tumorigenesis. *Mdm2*-null mice die at embryonic day (E) 3.5 prior to implantation,

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while *Mdm4*-deficient mice arrest at pre-gastrulation and die at E7.5. These embryonic lethal phenotypes are completely rescued by deletion of *p53* (3-5). Moreover, deletion of either *Mdm2* or *Mdm4* in neural progenitor cells of mouse is neonatal lethal. *Mdm2* loss in the brain results in hydranencephaly due to apoptosis, whereas *Mdm4* deficiency causes porencephaly due to both apoptosis and cell cycle arrest. Both phenotypes are rescued by deletion of *p53*. Interestingly, concomitant deletion of *Mdm2* and *Mdm4* in developing central nervous system (CNS) resulted in a more severe phenotype compared to loss of each gene alone, revealing that these proteins are not functionally redundant (6). In addition, while *Mdm2*^{+/-} or *Mdm4*^{+/-} mice have a normal life span, both are radiosensitive and, upon DNA damage, succumbed to p53-dependent early death (7). Furthermore, *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 (7). Lastly, conditional deletion of *Mdm2* in adult mice is also lethal due to extensive p53-induced irreversible tissue damage (8, 9), whereas deletion of *Mdm4* under similar condition causes reversible pathologies and is compatible with life (10). Many human tumors have high levels of MDM2 and MDM4 due to either gene amplification or overexpression. For instance, elevated level of MDM4 are detected in retinoblastoma (11, 12), melanoma (13), breast cancer (14, 15), glioblastoma (16) and hematopoietic neoplasms such as pre-B acute lymphoblastic leukemia (ALL) in adults (17) and acute myeloid leukemia (AML) (18). The major role of these proteins in tumor development and progression is thought to be inhibition of p53 activity as a large number of tumors with high MDM4 levels retain wild type *p53*. Mdm4 exerts its inhibitory effect via physical masking of the transactivation (TA) domain of p53 protein, and, unlike Mdm2, appears to have no direct role in controlling the level of p53 protein by degradation.

As a member of the p53 family, p73 is a transcription factor with substantial structural and functional homology to p53. Having the highest homology in the DNA binding domain (63%) as well as identical residues that interact with DNA, allows p73 to bind to the consensus p53-binding site, activates common target genes and mediate apoptosis and cell cycle arrest (19). However, unlike *p53*, *p73* is mutated in less than 0.5% of human tumors (20). Other studies have shown that *p73* is altered in various tumor types through loss of heterozygosity (LOH) or epigenetic silencing in tumors such as lymphoma and leukemia (21-24), glioma (25), neuroblastoma (26) and breast cancer (27). Such observations imply p73 functions as a tumor suppressor. In fact, genetic studies clearly highlight this function as *p73*^{+/-} mice develop spontaneous tumors (28). Also the combination of *p73* loss with loss of other tumor suppressor genes such as *p53* or presence of an oncogene such as *Myc* enhances tumor burden and/or increases tumor dissemination and metastasis (28-30). Although both p53 and p73 belong to the same family, distinct differences between them have been identified. For instance, genetic studies revealed that while *p53*-null mice are for the most part viable without prominent developmental defects, *p73*-deficient mice have serious developmental abnormalities such as chronic infections and inflammation, infertility due to abnormalities in pheromone signaling, and neurological defects including hippocampal dysgenesis, cortical hypoplasia and hydrocephaly (31).

Given the significance of Mdm2 and Mdm4 in regulating p53 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. Co-expression of *Mdm2* and *p73* in 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 (32-34). Such binding reduces p73 transcriptional activity (33, 35) and, in turn, *p73*-induced apoptosis (32). However, p53 and p73 bind to separate regions on Mdm2 (35) and, unlike p53, p73 is not destabilized or degraded by Mdm2 (32, 33). Moreover, recently, an *in vivo* study proposed that Mdm2 exerts its oncogenic activity through inhibition of both p53 and p73 functions (36). Mdm4, like Mdm2, also similarly binds p73 (34); 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 (37). To interrogate the biological significance of the Mdm4-p73 interaction *in vivo*, here, we present a comprehensive genetic characterization of the potential Mdm4 and p73 interaction during both development and tumorigenesis.

Materials and Methods

Mice

p73^{+/-} mice (28) (from Dr. Elsa Flores, MD Anderson Cancer Center, Houston, TX), *Mdm4*^{2/+} mice (5) and *p53*^{+/-} (38) mice on a mixed background (129/SvJae and C57BL/6) were used for our studies. *p73*^{+/-} mice (28), *Mdm4*^{fl/+} mice (5) and *Nes-Cre* transgenic mice (from Jackson laboratory) on C57BL/6 background were used for studying CNS development. *Mdm4*^{Tg15} mice (39) and *p73*^{+/-} mice (28), both on C57BL/6 background, were crossed to generate 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. Mice were sacrificed upon morbidity or reaching the 2-year endpoint of the study.

Histology and Immunohistochemistry

Whole embryos were fixed in 4% paraformaldehyde and paraffin embedded for sagittal sectioning. Collected organs were fixed in 10% formalin and paraffin embedded for sectioning. Sections were dewaxed and rehydrated according to standard protocols and stained with hematoxylin and eosin (H&E). 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).

Real-Time qRT-PCR

Total RNA was isolated from embryonic brain at E14.5 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase I (Roche Life Science, Indianapolis, IN, USA), and then reverse transcribed by using First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA). Quantitative real-time PCR was performed according to the manufacturer's instructions (Bio-Rad, Valencia, CA, USA). Expression was normalized to *Rplp0*.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6 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 followed by Newman-Keuls multiple comparison test. 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.

Results

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

Given that *Mdm4* is essential for dampening *p53* activity during embryogenesis and that *Mdm4* binds to *p73* with relatively higher affinity compared to *p53* in *in vitro* studies, we first examined whether the *Mdm4*-*p73* axis is critical for embryonic development. We intercrossed *Mdm4*^{2/+} (the *Mdm4*² allele represents a true null allele as opposed to the *Mdm4*⁻ allele which deletes exons 3-5 and leads to production of a small protein; see reference (2)) and *p73*^{+/-} mice to obtain *Mdm4*^{2/2} *p73*^{-/-} embryos and examine whether *Mdm4*^{2/2} embryonic lethality at E7.5 could be rescued in a *p73*-null background. To overcome the issue of *p73*^{-/-} infertility, we used *p73* heterozygous mice as breeders and consequently, the frequency of *Mdm4*^{2/2} *p73*^{-/-} embryos was expected to be 6.25%. At E11.5, we collected 36 embryos with normal morphology, 2 resorbed embryos and 16 empty deciduae. We did not obtain any *Mdm4*^{2/2} *p73*^{-/-} embryo with normal morphology; however, one of two resorbed embryos was *Mdm4*^{2/2} *p73*^{-/-} and the other was *Mdm4*^{2/2}. The phenotypes of both were comparable, indicating that *p73* loss does not rescue *Mdm4*^{2/2} phenotype at this developmental stage. Further, we studied the rescue at an earlier time point, 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 (*Mdm4*^{2/2} (1), *Mdm4*^{2/2} *p73*^{+/-} (1) and *Mdm4*^{2/2} *p73*^{-/-} (3) embryos). Additionally, we observed no significant difference in the morphology of *Mdm4*^{2/2} *p73*^{-/-} compared to *Mdm4*^{2/2}. Combined, *Mdm4*^{2/2} embryos, regardless of the *p73* genotype, were remnants of resorbed embryo with no recognizable morphology (Table 1) ($\chi^2=30.000$, *df*=5, *p*-value < 0.0001), indicating that loss of *p73*, in the presence of unrestricted *p53* activity, does not rescue the *Mdm4*-null phenotype.

***p53* haploinsufficiency does not contribute to the rescue of the *Mdm4*^{2/2} *p73*^{-/-} phenotype**

We next examined the contribution of both *p53* and *p73* to the rescue of *Mdm4*^{2/2} early embryonic lethality. We examined the role of *p53* gene dosage in the rescue of *Mdm4*^{2/2} early embryonic lethality and the effect of *p73* loss on the phenotype of *Mdm4*^{2/2} *p53*^{-/-} embryos. We crossed *Mdm4*^{2/2} *p53*^{-/-} with *Mdm4*^{2/+} *p73*^{+/-} mice to test whether 50% reduction in *p53* gene dosage extends the lifespan of *Mdm4*^{2/2} embryos and concomitantly whether deletion of one allele of *p73* suffices to further advance embryonic development. At E11.5, we observed 10 *Mdm4*^{2/+} *p53*^{+/-} embryos with normal morphology as well as 17 *Mdm4*^{2/2} *p53*^{+/-} embryos with an aberrant developmental phenotype, suggesting that deletion of one allele of *p53* cannot completely rescue the *Mdm4*^{2/2} phenotype. However, decreased *p53* dosage in *Mdm4*^{2/2} *p53*^{+/-} embryos did delay the *Mdm4*-null phenotype. Although these embryos were still severely runted compared to *Mdm4*^{2/+} *p53*^{+/-} embryos (Supplementary Figure S1a-c), they overcame pre-gastrulation arrest, as evidenced by the formation of neural groove with neural folds on each side as well as allantois, a conspicuous landmark of gastrulation (40). The observation of a partial rescue due to haploid loss of *p53* is in agreement with findings from another *Mdm4* knockout mouse model (41). Moreover, *Mdm4*^{2/2} *p53*^{+/-} and *Mdm4*^{2/2} *p53*^{+/-} *p73*^{+/-} embryos had comparable phenotypes, indicating that loss of one allele of *p73* is not sufficient to rescue *Mdm4*^{2/2} *p53*^{+/-} phenotype (Supplementary Table S1) ($\chi^2=10.000$, $df=3$; $p\text{-value}=0.0186$).

To determine whether the complete loss of *p73* can extend the survival of *Mdm4*^{2/2} *p53*^{+/-} embryos, we crossed *Mdm4*^{2/2} *p53*^{-/-} *p73*^{+/-} with *Mdm4*^{2/+} *p73*^{+/-} mice and analyzed embryos at E9.5. We obtained 18 *Mdm4*^{2/+} *p53*^{+/-} embryos, which were morphologically comparable to wild type, and 28 *Mdm4*^{2/2} *p53*^{+/-} embryos, which were abnormally small (Table 2) ($\chi^2=30.000$, $df=5$, $p\text{-value}<0.0001$). The *Mdm4*^{2/2} *p53*^{+/-} embryos in the background of wild type or null *p73* had the same phenotype (Supplementary Figure S1d-f), suggesting that *p73* loss has no impact on the *Mdm4*^{2/2} phenotype.

Loss of *p73* does not rescue the *Mdm4*^{2/2} embryonic brain phenotype

Given the strong dependence of *Mdm4* loss on *p53* activity in early development, we next focused on the possible cooperation of *Mdm4* and *p73* during development of the brain, an organ wherein both *Mdm4* and *p73* have well-established roles during embryogenesis. *Mdm4* plays an important role in neural development by regulating *p53* (41). Conditional deletion of *Mdm4* in the developing CNS by *Nestin*-driven *Cre recombinase* (*Nes-cre*) starts as early as E10.5 (6, 42) and 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 rescued by deletion of *p53* (6) (43). On the other hand, *p73* is well known as a multifunctional protein in the field of neurobiology (44) and crucial for the maintenance of neural stem cells (45, 46). *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 Cajal-Retzius (CR) cells at E12.5 (31, 47). However, the phenotype of *p73*-deficiency in the brain is not evident during prenatal life, except mild cortical hypoplasia after E14.5, and the core triad, including cortical

hypoplasia, hippocampal dysgenesis and ventriculomegaly, is only prominent in postnatal and adult mice (48).

Given that both Mdm4 and p73 have established roles in embryological development of 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 could be rescued by p73 loss. For this study, we used the *Mdm4^{fx}* conditional allele (5). We crossed *Mdm4^{fx/fx} p73^{+/-}* mice with *Mdm4^{fx/+} 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 (6). In total, 121 embryos were collected (Supplementary Table S2). The histopathological studies showed that the phenotype of porencephaly in *Mdm4^{fx/fx} Nes-cre* (n=3) and *Mdm4^{fx/fx} p73^{-/-} Nes-cre* (n=5) embryonic brains was comparable (Figure 1a–c). 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 1d–f and 1g–i). These results clearly show that even in a tissue wherein p73 has prominent functions, deletion of p73 does not rescue the *Mdm4*-null phenotype.

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 p73 specific target genes in various cell types or cancer cell lines under various conditions. In each set of experiments, different target genes have been identified (46, 49, 50). Thus, we decided to examine, in our experimental model system, whether p73 target genes known to play a role in the CNS were aberrantly expressed. Thus the embryos with the following genotypes were used for 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^{2/2}* embryonic brain), *Mdm4^{fx/fx} p73^{-/-} Nes-cre* (*Mdm4^{2/2} p73^{-/-}* embryonic brain). The *Mdm4^{2/2} p53^{-/-}* embryonic brains at E14.5 were obtained from intercrossing *Mdm4^{2/2} p53^{-/-}* mice.

14-3-3σ is a p53 target gene, which inhibits the G2/M progression and induces cell cycle arrest (51). Overexpression of *p73* upregulates the transcript of *14-3-3σ* two to six times higher than does *p53* overexpression (49, 50). p73 also directly binds to the consensus p53/p73-binding site in the regulatory region of *14-3-3σ* gene (49) suggesting that *14-3-3σ* might be a *bona fide* target of p73. More importantly, a comprehensive study on the differential expression of 14-3-3 protein isoforms showed that 14-3-3σ is located in the nuclei of developing rat hippocampus neurons (52) as well as human hippocampus (53), corresponding to the region that *p73^{-/-}* and *TAp73^{-/-}* mice display brain defects. In our analyses, *14-3-3σ* was highly expressed in *Mdm4^{2/2}* 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 2a), indicating that both p53 and p73 regulate *14-3-3σ*.

Perp is another p53 target gene, which mediates apoptosis (54) in a cell context-dependent manner (55). More specifically, studies on *Perp*-deficient mice have shown that *Perp* exerts its p53-dependent apoptosis in developing CNS as well as thymocytes; however, it is dispensable for apoptosis in E1A-expressing MEFs (55). Four p53 binding sites have been recognized within the promoter and the first intron of the murine *Perp* gene (56) and it had been suggested that p73 might bind to these sites as well (57). Thus, we examined the effect of *Mdm4* loss on *Perp* expression in the presence and absence of p53 or p73. *Perp* was significantly upregulated in *Mdm4*^{2/2} embryonic brain compared to wild type (*p*-value < 0.01), while loss of either p53 or p73 abolished its expression (*p*-value < 0.01 and <0.05, respectively) (Figure 2b).

p21 is the well-characterized canonical transcriptional target of p53 (58, 59) and a major mediator of p53-dependant cell cycle arrest. It was also recognized as one of the first p73 target genes in cells overexpressing wild type p73; yet, unlike p53, p73 does not induce *p21* in response to DNA damage (60). Moreover, the level of p21 induced by p53 was three to six times higher than p73 (50). In our system, *p21* was only upregulated in *Mdm4*-deficient embryonic brain in a p53-dependent manner (*p*-value < 0.01) (Figure 2c).

We also examined a number of p73 specific target genes, which modulate neurogenesis, including *p57* (61, 62), *Jag1* (63), *Jag2* (46, 49, 63), *Hes5* (45, 46) and *Notch2* (46); however, we 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 other pathways in the developing CNS.

Dampened p53 function overshadows the cooperation of Mdm4 and p73 during tumorigenesis

Given that overexpression of *Mdm4* and loss of p73 have been implicated in tumor development, we explored whether these two alterations cooperate during tumorigenesis. We generated a cohort of *Mdm4*^{Tg15} p73^{+/-}, *Mdm4*^{Tg15}, p73^{+/-} and wild type mice in C57BL/6 background. Since p73^{-/-} mice die at an early age due to malnutrition and growth retardation rather than tumor development, we did not include *Mdm4*^{Tg15} p73^{-/-} and p73^{-/-} mice in the cohort. *Mdm4*^{Tg15} is one of three generated *Mdm4* transgenic mouse lines which develop spontaneous tumorigenesis (39). At the two-year endpoint of this study, 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 curves of *Mdm4*^{Tg15} p73^{+/-} and *Mdm4*^{Tg15} mice was not statistically significant (Figure 3a). We 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 tumors 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

3b) (Table 3). Unlike $p73^{+/-}$ tumors that show approximately 40% LOH (36), none of the tumors from $Mdm4^{Tg15} p73^{+/-}$ mice underwent LOH (data not shown), suggesting that overexpressed Mdm4 might be inhibiting the $p73$ wild type allele in tumorigenesis. Intriguingly, four $Mdm4^{Tg15} p73^{+/-}$ mice had unclassified large cell brain tumor compared to one $Mdm4^{Tg15}$ mouse (20% of $Mdm4^{Tg15} p73^{+/-}$ mice compared to 6.6% of $Mdm4^{Tg15}$ mice). 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). These $Mdm4^{Tg15} p73^{+/-}$ mice also had several disseminated foci of lymphoma in lymphoid and non-lymphoid organs and/or multiple primary tumors (Figure 4). 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 (21, 22, 30), increased lymphomagenesis in $Mdm4^{Tg15} p73^{+/-}$ mice indicates a possible cooperation between Mdm4 and $p73$. Yet, these differences were not statistically significant perhaps due to the small sample size (Figure 3c).

DISCUSSION

The observations that Mdm4 is overexpressed in many tumors and most tumors lack $p73$ mutations, suggest overexpression of Mdm4 may be a mechanism for inactivation of $p73$ in human cancer. Despite the strong *in vitro* evidence of Mdm4- $p73$ binding in mammalian cells and the higher affinity of Mdm4 for $p73$ rather than $p53$ in biochemical studies, thus far no studies have examined the biological significance of this interaction. In this study, using genetic mouse models, we provide the first comprehensive *in vivo* characterization of the Mdm4 and $p73$ interaction during development and tumorigenesis.

The Mdm4- $p73$ interaction during development

Our results reveal that loss of $p73$ does not rescue $Mdm4$ -deficient early embryonic lethality, indicating that in the presence of enhanced $p53$ activity, such interaction does not have biological importance in embryogenesis. We further examined the consequences of the Mdm4- $p73$ interaction in the developing CNS, a more relevant biological system. We found that Mdm4 inhibits $p73$ -mediated up-regulation of *I4-3-3 σ* and *Perp*, indicating that $p73$ contributes to the $Mdm4$ -deficient brain phenotypes. However, the effect of unrestricted $p53$ and subsequently, the activation of a plethora of target genes are stronger than the effect of $p73$ and dominate the $Mdm4^{2/2} p73^{-/-}$ brain phenotype. Another explanation for the dominant effect of $p53$ activity is its expression pattern compared to $p73$ during CNS development. At E10.5, the expression of $p53$ is ubiquitous in the mouse brain (64) while $p73$ is expressed sparsely and mostly in the telencephalon (47). At later embryonic stages, $p53$ 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 (64), whereas $p73$ is highly expressed in cortical hem, a structure close to the hippocampus, and more specifically in CR cells (47). 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, $p53$ expression in more regions of brain dictates the $Mdm4^{2/2} p73^{-/-}$ brain phenotype.

Congenital porencephaly is a rare anomaly in the neonate's brain due to aberrant development of CNS (65). It is characterized as a cavity in cerebral hemispheres and causes severe seizures and mental retardation in patients (65). It is mostly sporadic and has been related to trauma and ischemic injury at mid-gestation (66), but the observations of familial porencephaly suggested genetics could also be involved in a subset of patients (67). Thus far, dysregulation of the Mdm4-p53 pathway has not been studied directly. However, *COL4A1* is among the limited mutations that have been found to be associated with this anomaly (68-70). Surprisingly, this gene is regulated by p53 as well as p73 (71, 72), which indicates that p53 and p73 pathway could be activated in porencephaly. Interestingly, hippocampal atrophy has been reported in 95% of cases with congenital porencephaly (73). Such striking coexistence implies a common mechanism underlying both pathologies; however, it remains 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 brains, the Mdm4-p73 interaction in developing CNS, and the prominent role of p73 in the formation of the hippocampus suggest that it is worth examining the genetic alterations of *Mdm4* and *p73* in the subset of patients with porencephaly and hippocampal atrophy.

The Mdm4-p73 cooperation during tumorigenesis

Our tumor study revealed that either Mdm4 overexpression or *p73* heterozygosity is tumorigenic which is consistent with previous studies (28, 39). *Mdm4^{Tg15} p73^{+/-}* mice also developed spontaneous tumors, a higher frequency of lymphoma and brain tumors, as well as a strikingly highly disseminated lymphoma compared to each individual alteration. *Mdm4^{Tg15}* mice in our cohort had longer survival and developed tumors with increased latency compared to our published *Mdm4^{Tg15}* cohort study (39). 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 (39). On the other hand, *p73^{+/-}* mice in our cohort had prolonged survival along with a very low frequency of tumorigenesis. Such a discrepancy between *p73^{+/-}* mice in our cohort and published studies could be due to the genetic background, a factor that has already been shown to affect the rate and spectrum of tumor development in other mouse models such as *p53^{-/-}* mice (74, 75). The *p73^{+/-}* mice in our cohort were in C57BL/6, whereas in the previous study these mice had mixed genetic background of C57BL/6 and 129/SvJ (28). Together, *p73* heterozygosity in C57BL/6 background develops tumors with lower frequency compared to mixed background, whereas Mdm4 overexpression develops tumor, yet the rates of lymphoma and brain tumor are very low. Interestingly, 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). However, the differences in survival curve and tumor spectrum of *Mdm4^{Tg15} p73^{+/-}* and *Mdm4^{Tg15}* mice 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. Our published study on *Mdm4* transgenic mouse lines clearly demonstrate that *Mdm4^{Tg1} p53^{+/-}* mice have significantly shorter survival compared to *Mdm4^{Tg1}* and *p53^{+/-}* mice while they have a longer life span than *p53^{-/-}* mice (39). In addition, tumors from *Mdm4^{Tg1} p53^{+/-}* mice showed a very low rate of LOH (less than 8%) compared to *p53^{+/-}* tumors with approximately 50% rate of LOH (76) signifying that the observed difference in the tumorigenesis of *Mdm4^{Tg1} p53^{+/-}* mice results from cooperation of Mdm4 and p53 and ultimately dampened p53 activity (39). The comparison of *Mdm4^{Tg15} p73^{+/-}*, *Mdm4^{Tg1} p53^{+/-}*, *Mdm4^{Tg15}* and *Mdm4^{Tg1}* survival curves of current and previously published studies (39) clearly show that the *Mdm4^{Tg1} p53^{+/-}* survival curve is significantly shifted to the left compared to *Mdm4^{Tg15}* and *Mdm4^{Tg1}* survival curves, whereas the survival curve of *Mdm4^{Tg15} p73^{+/-}* is barely shifted to the left (Figure 3c). Since the survival and tumor spectrum of *Mdm4^{Tg15} p73^{+/-}* mice are not significantly different compared to those of *Mdm4^{Tg15}*, our data underscore the strength of dampened p53 activity in driving the tumor phenotype in this model, which dominates the possible cooperation between Mdm4 and p73 during tumorigenesis.

Supplementary Material

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Figure 1.

Loss of *p73* does not rescue the *Mdm4*-null porencephaly phenotype. Representative sagittal sections of embryos at E14.5. 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.

Figure 2.

Deletion of *Mdm4* results in the upregulation of some *p73* and *p53* common target genes in embryonic brain. Real-time RT-qPCR was performed for *14-3-3σ* (a), *Perp* (b), *p21* (c) 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 comparison test. Error bars:SEM.

Figure 3.

(a) Kaplan-Meier survival curves of $Mdm4^{Tg15} p73^{+/-}$, $Mdm4^{Tg15}$, $p73^{+/-}$ and wild type mice are shown. (b) The tumor spectrum of $Mdm4^{Tg15} p73^{+/-}$, $Mdm4^{Tg15}$ and $p73^{+/-}$ mice. (n indicates the total number of tumors). (c) Kaplan-Meier survival curves of $Mdm4^{Tg15} p73^{+/-}$, $Mdm4^{Tg15}$, and published data of $Mdm4^{Tg1}$, $Mdm4^{Tg1} p53^{+/-}$ mice. The number of animals per genotype is indicated.

Figure 4.

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 effaced by lymphoma cells) (i). The second primary tumor is sarcoma in colon (e). Magnification, $\times 4$.

Table 1

p73 loss does not rescue the *Mdm4*-null embryonic lethality Cross: $Mdm4^{+/-} p73^{+/-} \times Mdm4^{+/-} p73^{+/-}$

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> ^{+/-} <i>p73</i> ^{+/+}	6	9.125	8.219	12.5
<i>Mdm4</i> ^{+/-} <i>p73</i> ^{+/-}	32	18.25	48.836	25
<i>Mdm4</i> ^{+/-} <i>p73</i> ^{-/-}	16	9.125	21.918	12.5
<i>Mdm4</i> ^{2/-} <i>p73</i> ^{+/+}	0*	4.5625	0	6.25
<i>Mdm4</i> ^{2/-} <i>p73</i> ^{+/-}	0*	9.125	0	12.5
<i>Mdm4</i> ^{2/-} <i>p73</i> ^{-/-}	0*	4.5625	0	6.25
Total	73¶	73	100	100

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

¶ Sixteen empty deciduae were also collected.

Table 2

p73 loss does not prolong the partial rescue of *Mdm4*^{2/2} *p53*^{+/-} embryos Cross: *Mdm4*^{2/2} *p53*^{-/-} *p73*^{+/-} × *Mdm4*^{+/-} *p73*^{+/-}

Genotype	Number of E9.5 embryos		Percentage of embryos	
	Observed	Expected	Observed	Expected
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	1	2.25	5.55	12.5
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	14	4.5	77.78	25
<i>Mdm4</i> ^{+/-} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	3	2.25	16.67	12.5
<i>Mdm4</i> ^{2/2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/+}	0 [§]	2.25	0	12.5
<i>Mdm4</i> ^{2/2} <i>p53</i> ^{+/-} <i>p73</i> ^{+/-}	0 [§]	4.5	0	25
<i>Mdm4</i> ^{2/2} <i>p53</i> ^{+/-} <i>p73</i> ^{-/-}	0 [§]	2.25	0	12.5
Total	18 [¶]	18	100	100

[§] At E9.5, seven *Mdm4*^{2/2} *p53*^{+/-} *p73*^{+/+}, 16 *Mdm4*^{2/2} *p53*^{+/-} *p73*^{+/-} and 5 *Mdm4*^{2/2} *p53*^{+/-} *p73*^{-/-} developmentally retarded embryos were observed.

[¶] Six empty deciduae were also collected.

Table 3Spontaneous tumor spectrum of *Mdm4^{Tg15} p73^{+/-}*, *Mdm4^{Tg15}* and *p73^{+/-}* mice

Tumor type	Genotype		
	<i>Mdm4^{Tg15} p73^{+/-} a</i> (n=18) [#]	<i>Mdm4^{Tg15} b</i> (n=14) [#]	<i>p73^{+/-} c</i> (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 moribundity. Sixteen of them are 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 moribundity. Fourteen of them are diagnosed with tumor and one mouse had two primary tumors. Among 2 mice sacrificed due to 2-year time point, one mouse were diagnosed with tumor.

^c Among 13 *p73^{+/-}* mice, one mouse was sacrificed due to moribundity 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 with lymphoma (infiltrated on 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