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Guilty as CHARGED: p53's expanding role in disease

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Unrestrained p53 activity during development, as occurs upon loss of the p53 negative regulators Mdm2 or Mdmx, causes early embryonic lethality. Surprisingly, co-expression of wild-type p53 and a transcriptionally-dead variant of p53, with mutations in both transactivation domains ($p53^{L25Q,W26S,F53Q,F54S}$), also causes lethality, but later in gestation and in association with a host of very specific phenotypes reminiscent of a syndrome known as CHARGE. Molecular analyses revealed that wild-type p53 is inappropriately activated in $p53^{5,26,53,54/+}$ embryos, triggering cell-cycle arrest or apoptosis during development to cause CHARGE phenotypes. In addition, CHARGE syndrome is typically caused by mutations in the CHD7 chromatin remodeler, and we have shown that activated p53 contributes to phenotypes caused by CHD7-deficiency. Together, these studies provide new insight into CHARGE syndrome and expand our understanding of the role of p53 in diseases other than cancer.

Keywords: p53, transcriptional activation, CHARGE syndrome, CHD7, embryonic development, Ribosomopathy, Mdm2, neural Crest

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Introduction

The p53 protein plays a fundamental role in suppressing cancer, as evidenced by the findings that p53 is mutated in over half of human tumors and that *p53* null mice display a completely penetrant cancer predisposition.[1-4] The ability of p53 to restrain malignancy is thought to relate to its ability to respond to a wide spectrum of cellular stresses by inducing cell-cycle arrest or apoptosis to limit expansion of neoplastic cells.[5,6] p53 is a transcriptional activator, which binds to specific DNA elements throughout the genome, leading to the induction of p53 target genes involved in the apoptosis or cell-cycle arrest programs, although p53 can also act through transactivation-independent mechanisms such as direct induction of apoptosis through cytoplasmic interactions with Bcl-2 family members.[7,8] In addition to an unequivocal role in cancer suppression, the ability of p53 to respond to cellular stresses has broader significance, as p53 participates in additional physiological and pathological processes. For example, p53 activation drives some of the deleterious phenotypic effects of stroke, neurodegenerative disease, and genotoxic cancer therapies.[9,10] Thus, p53 activity can be beneficial or detrimental, depending on the context, underscoring the importance of keeping it tightly controlled.

Keeping the Brakes on p53

The critical importance of restraining p53 in a physiological context was originally appreciated through analyses of knockout mice deficient for the p53 negative regulators Mdm2 and Mdmx.[11-16] Mdm2 binds and inhibits p53's 2 transcriptional activation domains (TADs) as well as acting as an E3 ubiquitin ligase to promote p53 degradation,[17,18] while Mdmx binds and inhibits the p53 TADs but only affects stability through interactions with Mdm2 rather than ubiquitylating p53 itself.[19-21] Loss of Mdm2 in mice causes early embryonic lethality at ~E3.5, attributable to inappropriate p53 activity, as evidenced by the complete viability of $Mdm2^{-/-};p53^{-/-}$ mice. These observations provided the first demonstration that unrestrained p53 activity during development can drive embryonic lethality.[13,15,16] Similarly, $Mdmx^{-/-}$ mice are

also embryonic lethal, although at a later timepoint (between E7.5–11.5), and this lethality is again fully rescued by p53 deficiency.[11,12,14] Subsequent studies using *Mdm2* and *Mdmx* conditional mouse strains further revealed the necessity of regulating p53 in various tissues, including the heart, haematopoietic system, skin, bone, and neuroepithelium, where p53 activation causes increased cell-cycle arrest, senescence or apoptosis and perturbs proper tissue development.[19,22-29] Thus, the appropriate restraint of p53 is fundamental for proper embryo and tissue development.

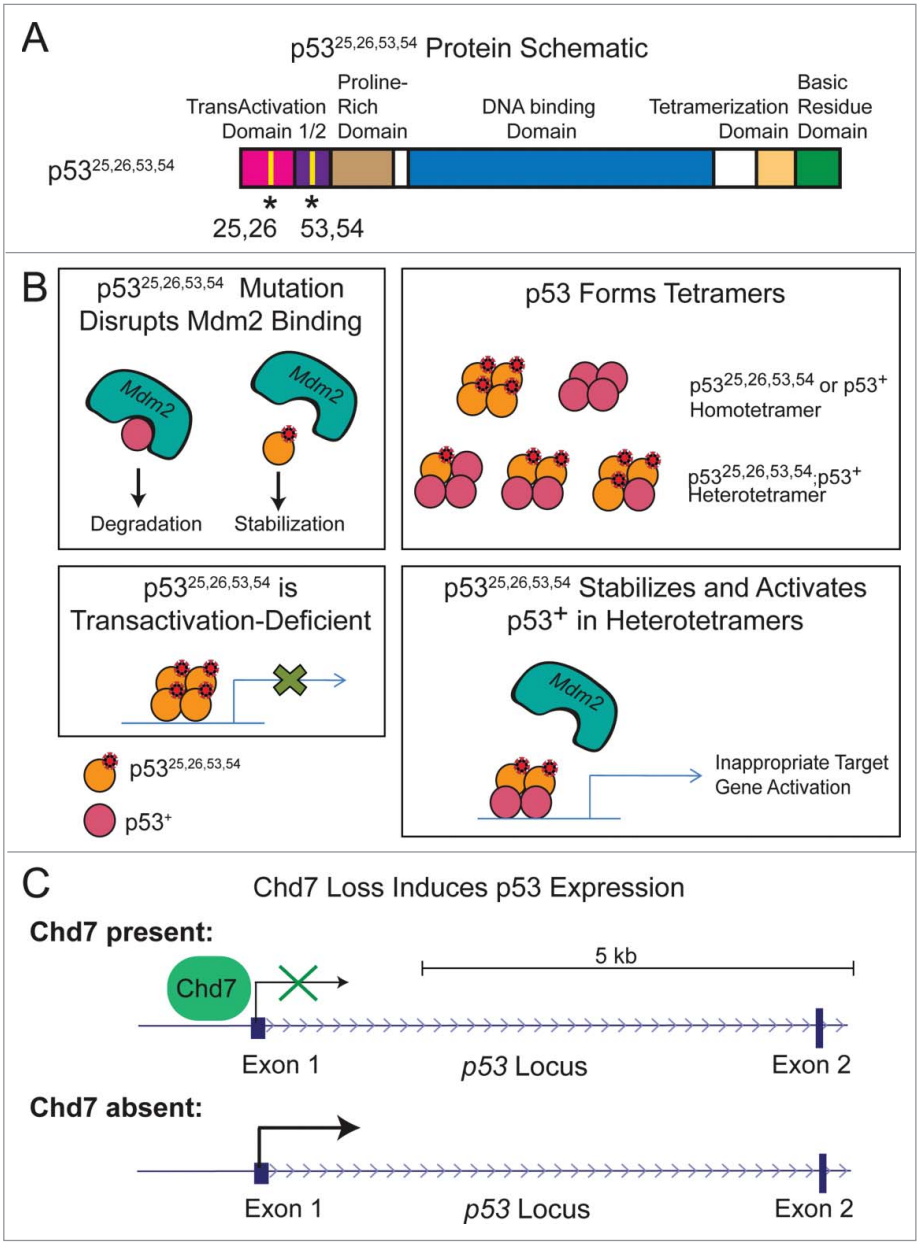

Figure 1. p53 is induced in *p53*$^{25,26,53,54/+}$ embryos and *Chd7*-null cells. (**A**) Schematic of the p53^{25,26,53,54} protein with L25Q, W26S, F53Q, and F54S mutations and structural domains indicated. (**B**) Model for how p53^{25,26,53,54} may stabilize and activate wild-type p53 (denoted by p53^{+}). Top Left: p53^{25,26,53,54} mutations disrupt the p53-interaction with Mdm2, resulting in stabilized p53 protein. Top Right: p53 can form tetramers comprised of any combination of mutant and/or wild-type p53 proteins, which can result in homotetramers or heterotetramers. Bottom Left: p53^{25,26,53,54} can bind to DNA but is transactivation-deficient. Bottom Right: p53^{25,26,53,54} can interact with wild-type p53 in heterotetramers, disrupt the p53-interaction with Mdm2, and promote wild-type p53 to transactivate target genes. Shown is an example of the type of heterotetramer that can form, although other subunit compositions are possible. **C)** Chd7 loss induces *p53* expression. One mechanism by which Chd7 loss could induce p53 expression is through loss of Chd7 binding to the *p53* promoter, resulting in derepression of *p53* expression.

Elucidating the Role of Transactivation in p53-driven Developmental Phenotypes

We sought to follow-up on these studies by investigating the mechanisms through which p53 activation during embryogenesis promotes developmental phenotypes. In particular, given the various biochemical activities of p53, we set out to address the requirement for p53 transcriptional activation potential in driving developmental phenotypes. Toward this end, we leveraged *p53* mutant knock-in mouse strains that we generated previously as tools to assess the contribution of p53 transactivation activity to different biological functions.[30,31] In these strains, mutations were introduced into the first (L25Q,W26S) or both (L25Q,W26S, F53Q,F54S) of p53's 2 amino-terminal TADs (**Fig. 1A**) to create the p53^{25,26} and p53^{25,26,53,54} mutants. These mutations were originally shown to compromise p53's transactivation activity in reporter assays *in vitro*, and we previously utilized these knock-in mice to study p53 transcriptional programs underlying responses to genotoxic cancer therapy and tumor suppression.[30-34] Using genome-wide expression profiling, we found that p53^{25,26} displays selective transactivation function, being severely compromised for the ability to activate most p53 target genes, but fully active on a subset of p53 target genes, while p53^{25,26,53,54} is completely transactivation-deficient (**Fig. 1B**).[30,35] Moreover, our studies revealed that the p53^{25,26,53,54} mutant is unable to promote apoptosis *in vivo* in response to gamma-irradiation or to suppress tumorigenesis in various mouse cancer models, indicating the transactivation function is critical for these biological processes.[30,36] Interestingly, the p53^{25,26} mutant showed separation-of-function activity, where it could not trigger

cell-cycle arrest or apoptosis in response to genotoxic damage but could suppress growth of a variety of cancers,[30,31,36] suggesting that the selective transcriptional activity manifested by this mutant accounts for its function in tumor suppression. Hence, we set out to similarly address the importance of transcriptional activation for p53-driven developmental phenotypes, using the same mutants. Importantly, the mutations at residues 25 and 26 disrupt the Mdm2-p53 interaction, resulting in stabilized p53 (**Fig. 1B**).[37] Thus, it is possible to examine the mechanism of p53-induced embryonic lethality using these mice, as Mdm2 binding and transactivation are both perturbed (although transactivation is compromised to different extents in the 2 strains). The p53 TAD mutant knock-in mice conditionally express *p53* upon deletion of an upstream *lox-stop-lox* cassette by the Cre recombinase, and therefore they were crossed to CMV-Cre mice, which ubiquitously express Cre.[38] Widespread expression of a single copy of $p53^{25,26}$, in $p53^{25,26/-}$ embryos, resulted in mid-gestational embryonic lethality, which we hypothesized reflected the ability of $p53^{25,26}$ to retain efficient transactivation of a subset of p53 target genes, including *Bax*, which had been shown to be partially responsible for the lethality in *Mdm2*-null embryos.[15] The importance of residual transactivation activity in triggering embryonic lethality in $p53^{25,26/-}$ embryos was confirmed upon evaluation of $p53^{25,26,53,54/-}$ embryos, where we found that widespread expression of a single copy of the transactivation-dead $p53^{25,26,53,54}$ mutant was compatible with the generation of viable adults. These data therefore reveal that p53 transactivation potential is essential for the embryonic lethality resulting from stabilized p53.

A Model for CHARGE Syndrome

In conducting these studies, we discovered, much to our surprise, that co-expression of the transactivation-dead $p53^{25,26,53,54}$ mutant and wild-type p53 triggers mid-late gestational embryonic lethality at ~E13.5–15.5.[35] This finding suggested a genetic interaction between wild-type and mutant p53, whose intricate nature we subsequently revealed by *in vitro* analyses.[35] We found that due to the disruption of the Mdm2-p53 interaction caused by mutation of the 25,26 residues, $p53^{25,26,53,54}$ is stabilized, which in turn allows it to interact with and stabilize wild-type p53 to adequately high levels for wild-type p53 to bind and transactivate select target genes and to induce apoptosis and cell-cycle arrest (**Fig. 1B**). However, the level of p53 activity in $p53^{25,26,5,354/+}$ embryos arising from the combination of a stable transactivation-dead mutant p53 protein with wild-type p53 is clearly not equivalent to the activity levels of wild-type p53 due to *Mdm2* loss, as demonstrated by the more moderate phenotypes in $p53^{25,26,53,54/+}$ embryos than in $Mdm2^{-/-}$ embryos.[13,15,16] Indeed, *Mdm2*-nullizygosity induces stabilized, fully active wild-type p53, while combined expression of wild-type p53 and $p53^{25,26,53,54}$ in $p53^{25,26,53,54/+}$ embryos merely causes modest p53 activation, as only wild-type p53 subunits are active while $p53^{25,26,53,54}$ subunits are transactivation-dead (**Fig. 1B**). Collectively, these findings were extremely surprising and completely unexpected, as transcriptionally-dead p53 molecules typically display dominant-negative activities. This study therefore serves as a novel paradigm for how other p53 mutant proteins, as well as potentially other mutant proteins, might drive disease phenotypes. Of note, the activation of wild-type p53 by the tumor suppression-dead $p53^{25,26,53,54}$ mutant suggests one reason for the lack of such transactivation-deficient mutants in human cancers.

Interestingly, the $p53^{25,26,53,54/+}$ embryos exhibited an exquisitely specific spectrum of phenotypes including coloboma of the retina (failure of optic closure), heart outflow tract and atrioventricular cushion defects, and inner and outer ear defects – a collection of phenotypes very reminiscent of a syndrome known as CHARGE.[39] CHARGE is an autosomal dominant congenital syndrome named for its characteristic features: ocular **c**oloboma, **h**eart defects, choanal **a**tresia (narrowing of the nasal passage), **r**etarded growth and development, **g**enitourinary hypoplasia, and **e**ar abnormalities (**Fig. 2**).[39,40] In 70–90% of cases, CHARGE is caused by mutations in *CHD7*, encoding an ATP-dependent chromatin remodeler.[41,42] However, while $Chd7^{+/-}$ mice exhibit some features of CHARGE, they have not been described to display major hallmarks, such as coloboma and heart outflow tract defects, and $Chd7^{-/-}$ embryos display embryonic lethality at E10.5.[43-45] In contrast, our $p53^{25,26,53,54/+}$ embryos survive until later in gestation and exhibit a full spectrum of CHARGE symptoms.[35] In fact, the manifestation of extensive ear malformations, coloboma, and heart defects in $p53^{25,26,\ 53,54/+}$ embryos emphasizes the value of our model for recapitulating a comprehensive form of CHARGE. In addition, while the embryonic lethality in mouse contrasts with human CHARGE, where individuals are mostly viable, it remains possible that fetuses with more severe phenotypes may die in utero, but this has not yet been demonstrated.[46]

A Role for p53 Downstream of Chd7

While these mouse studies show that p53 activation can cause features of CHARGE, the role of p53 downstream of Chd7 deficiency and in human CHARGE remained unclear. We therefore investigated whether there was any crosstalk between Chd7 and p53. Although we found that p53 activation did not affect Chd7 levels, we showed that the converse was true, that in several settings Chd7 loss triggers p53 activation.[35] First, as neural crest cells (NCCs) are thought to at least partially underlie CHARGE phenotypes,[39,47,49] we examined mouse NCCs and found that Chd7 inactivation resulted in increased expression of p53 and select p53 target genes (**Fig. 1C**). Second, to extend these data to human CHARGE patient samples, we examined p53 activity in fibroblasts from control and *CHD7*-mutation positive CHARGE patients. We found both elevated p53 protein and heightened p53 transcriptional activity on select p53 target genes in CHARGE patient fibroblasts relative to controls. Thus, in this human cell setting as well,

Phenotypes	CHARGE Syndrome	$p53^{25,26,53,54/+}$
Coloboma	Major	+
Heart Defects	Minor	+
Atresia of Choanae	Major	nd
Retardation of Growth	Minor	+
Genital Defects	Minor	nd
Ear Defects - Inner/Outer	Major	+
Cranial Nerve Abnormality	Major	nd
Cleft Palate/Lip	Minor	+
Tracheo-Esophageal Fistula	Minor	nd
Mandibular Hypoplasia	Other	+
Kidney Defects	Other	+
Bone/Cartilage Defects	Other	+
Thymus Aplasia	Other	+
Exencephaly	Not Reported	+
Responsible Gene	*Chd7*	*p53*

nd: not determined, in part due to embryonic lethality
Major Features: Very common in CHARGE syndrome but occur relatively rarely in other conditions
Minor Features: Occur with lower frequency or are less specific to CHARGE syndrome
Other Features: Rare, less specific phenotypes

(Blake, et al, 1998, 2006)

Figure 2. $p53^{25,26,53,54/+}$ embryos display phenotypes characteristic of CHARGE syndrome. Characteristics of CHARGE syndrome are considered major features, minor features, or other features, depending on the specificity to CHARGE and the utility of the feature for diagnosing CHARGE. The phenotypes contributing to the CHARGE acronym are shaded in gray. Phenotypes observed in $p53^{25,26,53,54/+}$ embryos are indicated with a +. nd: not determined in $p53^{25,26,53,54/+}$ embryos, due in part to mid-late gestational embryonic lethality.

CHD7 deficiency results in increased p53 levels and enhanced p53 target gene expression. Finally, using thymus samples from *CHD7*-mutation positive CHARGE syndrome patients, we observed increased p53 stabilization by immunohistochemistry in the thymi of fetuses from CHARGE patients relative to control fetuses. Collectively, these data suggest that CHD7 loss triggers p53 activation. However, to unequivocally implicate p53 in CHARGE, an examination of whether phenotypes manifesting with *Chd7* deficiency are rescued by *p53* deficiency was required.[44] Our analysis of embryonic phenotypes associated with *Chd7*-nullizygosity showed that they are partially rescued by *p53*-heterozygosity.[35] Specifically, the developmental delay, as assessed by somite number, was completely rescued, but limb and craniofacial formation were only partially rescued. Collectively, these findings indicate that p53 does indeed contribute to phenotypes downstream of Chd7 loss and strongly implicate p53 in promoting phenotypes in *CHD7* mutation-positive human CHARGE patients.

It is also possible that p53 could function independently of CHD7 to promote CHARGE phenotypes in the ~10–30% of cases where *CHD7* mutations have not been identified. For example, mutations in genes other than *CHD7* could trigger p53 activation. Alternatively, *p53* or components of the p53 pathway could be mutated in some CHARGE cases. Our mutational analysis of the *p53* coding region in a subset of CHD7-intact CHARGE patients did not reveal any *p53* mutations, although we analyzed a limited sample size.[35] Moreover, the modest levels of p53 activation that promote the CHARGE-like phenotypes in our mouse studies may be difficult to achieve by point mutation of the *p53* coding sequence. Instead, mutations in *p53* regulatory elements or in genes encoding key p53 regulators could have the effect of slightly activating p53 to promote CHARGE phenotypes and thus may be more likely to occur.[50] Collectively, our findings demonstrate a direct connection between Chd7 loss and p53 activation in CHARGE, highlighting the importance of our $p53^{25,26,53,54/+}$ mouse model for studying CHARGE.

Signaling from Chd Proteins to p53

The observation that p53 is activated upon Chd7 loss raises the question of the signal transduction cascade involved. Using chromatin immunoprecipitation (ChIP), we were able to show that Chd7 binds to the *p53* promoter in mouse NCCs and that Chd7 deficiency results in increased *p53* transcripts in both mouse NCCs and CHARGE patient fibroblasts, together suggesting that Chd7 directly represses p53 transcription (**Fig. 1C**).[35] However, Chd7 loss could also activate p53 indirectly, through more canonical pathways that impinge upon p53. It is well-established that p53 is activated post-translationally by stress signals, such as DNA damage or oncogene expression, via disruption of the p53-interaction with Mdm2 and consequent p53 stabilization.[6] Furthermore, defects in ribosomal biogenesis, such as those triggered by ribosomal subunit mutation, also activate p53 by perturbing the Mdm2-p53 interaction.[51] Interestingly, Chd7 has been shown to play a role in ribosome biogenesis, as

Chd7 binds to rDNA and enhances rRNA expression.[52] Chd7 inactivation results in both reduced pre-rRNA and global protein synthesis, suggestive of the existence of ribosome defects. In addition, Chd7 interacts with the nucleolar protein Treacle (encoded by *Tcof1*), which when mutated causes defects in ribosome biogenesis and p53 activation,[52,53] and it is thus quite plausible that Chd7 loss similarly triggers p53 activation at the post-translational level through this pathway.

Chd7 is a member of a larger CHD (chromodomain helicase DNA-binding) protein family, comprising 9 ATP-dependent chromatin remodelers (Chd1-9), some of which directly bind DNA.[54] Interestingly, several of these have previously been reported to impinge upon the p53 pathway.[55-57] Notably, loss of Chd8, which interacts with Chd7, causes early embryonic lethality that is partially rescued by p53 deficiency.[55,58] Chd8 was found to bind to p53 along with H1 to suppress p53 transactivation activity, thereby preventing p53-mediated apoptosis during embryogenesis.[55] In addition, Chd4 regulates p53 at the post-translational level by deacetylating p53, such that Chd4 loss enhances p53 acetylation and activity.[56] In addition, embryos with duplication of 1p36, which contains the tumor suppressor gene *Chd5*, exhibit enhanced senescence and apoptosis as well as perinatal lethality, which are rescued by p53 deficiency.[57] Activation of p53 in this context is attributable to Chd5 positively regulating $p19^{Arf}$ expression, which liberates p53 from Mdm2.[57] Therefore, the ability of Chd proteins to regulate p53 at the post-translational level has a strong precedent. Additional studies in the future will help to fully elaborate the molecular interactions between Chd7 and p53.

The Role of p53 in Other Diseases

Our findings that p53 plays a causative role in CHARGE syndrome phenotypes supports the emerging idea that p53 can have deleterious effects, contributing to diseases beyond cancer (**Fig. 3**). Support for this notion came originally from studies showing that p53-induced apoptosis in neurons contributes to the associated pathologies in stroke and neurodegenerative diseases, including Alzheimer, Parkinson and Huntington's diseases.[9] Subsequently, p53 activation was implicated in several genetic diseases, including Diamond Blackfan Anemia (DBA), 5q-syndrome, and Treacher Collins syndrome (**Fig. 3**).[53,59,61] Interestingly, these diseases are all considered ribosomopathies in which defects in ribosomal biogenesis are thought to underlie the observed symptoms. In DBA, mutation of genes encoding ribosomal proteins, such as *RPS19*, results in red blood cell anemia associated with developmental abnormalities, including orofacial, hand, limb, urogenital, and heart defects.[61] Mutation or deletion of *Rps19* in mouse models results in red blood cell anemia and skin hyperpigmentation, which are rescued by *p53* deletion.[60-62] In 5q-syndrome, patients carry a deletion of chromosome 5q, which includes the *Rps14* gene, and these patients exhibit macrocytic anemia, megakaryocyte dysplasia, and haematopoietic progenitor cell defects, phenotypes which can all be ameliorated by loss of p53 in mouse models.[59,61,63] Treacher Collins syndrome is caused by mutations in the *TCOF1* gene and is characterized by craniofacial defects such as mandibular hypoplasia, cleft palate, ear defects, and coloboma of the eye in humans, and p53 deficiency can rescue the neonatal lethality and craniofacial defects observed in *Tcof1* mutant mice.[53,64] In these ribosomopathies, deficiency in the implicated gene products is thought to compromise ribosome biogenesis, hence triggering p53 activation. An active contribution of p53 to these syndromes has been proposed based both on observations that p53 is stabilized in mouse and human patient tissues and rescue experiments in mouse models (**Fig. 3**).[53,59,60,65,66]

Beyond activation of p53 by the aforementioned stress cues, *p53* mutation may also lead to alterations in p53 activity, with pathological consequences (**Fig. 3**). This notion is exemplified by our mouse model, where coordinate expression of p53[25,26,53,54] and wild-type p53 is sufficient to drive CHARGE phenotypes.[35] In addition, mice homozygously expressing a p53 truncation mutant, $p53^{\Delta 31}$ – which lacks the C-terminal negative regulatory domain – develop dyskeratosis congenita, a syndrome characterized by nail dystrophy, oral leukoplakia, skin hyperpigmentation, aplastic anemia, and pulmonary fibrosis and attributable to telomerase dysfunction and consequent telomere attrition.[67,68] A direct role for p53 in triggering phenotypes upon telomere erosion is indicated by studies in telomerase-deficient mice, where germ cell depletion is partially rescued by *p53* loss.[69] While telomere attrition clearly induces DNA damage, thereby activating p53, some effects may also be through ribosome biogenesis defects, as telomere maintenance proteins are also required for processing of rRNAs.[70] Collectively, these data suggest that p53 contributes to developmental defects in ribosomal biogenesis syndromes. As mentioned above, it could be that CHD7 deficiency promotes CHARGE phenotypes by similarly triggering p53 activity through effects on ribosome biogenesis.

p53 is also implicated in diseases associated with defective NCC function (**Fig. 3**). Indeed, CHARGE is thought to arise at least in part from defects in NCC survival, proliferation, or migration.[47-49] Additionally, the 22q11 deletion syndromes, including Velocardiofacial syndrome and DiGeorge syndrome, in which patients present with heart defects, hypoplastic thymus, and mild craniofacial defects, are also thought to be partially attributable to defects in NCCs.[71,72] p53 suppression by genetic ablation or pharmacological inhibition can partially rescue phenotypes of *Tbx1* heterozygous mice, which develop features of DiGeorge syndrome, including cardiac outflow tract defects and thymic aplasia.[73] Furthermore, p53 has been reported to contribute to NCC defects in Treacher Collins syndrome, where p53 activation in $Tcof1^{+/-}$ embryos triggers apoptosis of neuroepithelial cells and deficiencies in the formation of migrating cranial NCCs, culminating in craniofacial malformations.[53] Finally, upon inactivation of the Pax3 transcription factor, in a mouse model for Waardenburg syndrome Type I/III (in which patients exhibit pigmentation defects of the eye, hair, and skin as well as hearing loss), p53-dependent apoptosis in migrating cardiac NCCs causes outflow tract septation defects.[74-77]

p53-Hyperactivity Syndromes	Phenotypes in Human Syndrome	Commonly Mutated Genes	p53 Mutation can Promote Disease Phenotypes in Mice	p53 Accumulation in Human Patient Samples/Cells	Phenotypes in Mouse Model for Disease Rescued by p53-Deficiency	References
CHARGE syndrome	Coloboma, Heart Defects, Choanal Atresia, Retardation of Growth, Genitourinary Defects, Ear Defects	*CHD7*	$p53^{25.26.53.54/+}$	+	$Chd7^{-/-}$	Van Nostrand, et al, 2014 Bergman, et al, 2011 Hurd, et al, 2007 Blake et al, 1998
Dyskeratosis Congenita	Nail Dystrophy, Oral Leukoplakia, Skin Hyperpigmentation, Aplastic Anemia, Pulmonary Fibrosis	Telomere Maintenance Genes: e.g. *DKC1, TERT*	$p53^{\Delta 31/\Delta 31}$	+	$Terc^{-/-}$	Simeonova, et al, 2013 Chin, et al, 1999 Pereboom, et al, 2011 Khincha & Savage, 2013
Diamond Blackfan Anemia	Macrocytic Anemia, Orofacial, Hand, or Limb Malformations, Heart Defects	Ribosomal genes: e.g. *RPS19*		+	$Rps19^{Dsk3/+}$	Boultwood, et al, 2012 McGowan, et al, 2008 Dutt, et al, 2011 Danilova, et al, 2008
5q-syndrome	Macrocytic Anemia, Megakaryocyte Dysplasia	Chr 5 deletion: e.g.*RPS14*		+	$Rps14^{+/-}$	Barlow, et al, 2010 Pellagatti, et al, 2010 Gaballa & Besa, 2014
Treacher Collins syndrome	Facial Hypoplasia, External Ear Defects, Coloboma	*TCOF1*			$Tcof^{+/-}$	Jones, et al, 2008 Kadakia, et al, 2014
Waardenburg syndrome TypeI/III	Skin, Hair, & Eye Pigmentation Defects, Sensorineural Hearing Loss, Dystopia Canthorum	*PAX3*			$Pax3^{-/-}$ (sp/sp)	Pani, et al, 2002 Morgan, et al, 2008 Wang, et al, 2011 Pingault, et al, 2010
DiGeorge syndrome	Heart Defects, Thymic Aplasia, Cleft Palate, Facial defects, Hypoparathyroidism	22q Deletion: e.g.*TBX1*			$Tbx1^{+/-}$	Caprio & Baldini, 2014 Gao, et al, 2013

Figure 3. p53 hyperactivity is associated with multiple syndromes. Syndromes associated with p53 hyperactivity identified using mouse models and human patient samples. The phenotypes observed in human patients and the most commonly mutated genes associated with each syndrome are noted. The genotypes of the mouse models are noted. Our work identifies p53 hyperactivity in CHARGE syndrome using 3 independent criteria: *p53* mutation being sufficient to cause phenotypes of the syndrome in a mouse model, p53 accumulation and/or activation in CHARGE patient samples, and *p53*-deficiency being able to rescue phenotypes in Chd7-deficient mice.

Whether defects in neural crest cells in $p53^{25,26,53,54/+}$ embryos are responsible for inducing the CHARGE-like phenotypes is unknown, but it will be interesting in future to determine the cell type(s) underlying phenotypes in $p53^{25,26,53,54/+}$ embryos. Collectively, these mouse models show that p53 activation can promote an ever-broadening range of diseases – a role for p53 that until recently has been greatly underappreciated.

p53 Hyperactivity in Mice: From Embryonic Lethality to Premature Aging

Interestingly, the collection of phenotypes in $p53^{25,26,53,54/+}$ embryos represents one in a range of mouse models where differing levels of p53 activity cause diverse phenotypes, ranging from embryonic lethality to premature aging (**Fig. 4**). Loss of Mdm2 or Mdmx results in early embryonic lethality stemming from high levels of activated wild-type p53.[11-16] In contrast, our $p53^{25,26,53,54/+}$ embryos display mid-late gestational embryonic lethality and CHARGE-like phenotypes, which are less severe than those observed upon Mdm2 or Mdmx loss.[35] The defects in $p53^{25,26,53,54/+}$ embryos are due to modest p53 activation resulting from mixed tetramers forming between wild-type p53 and transactivation-dead $p53^{25,26,53,54}$.

Another set of mouse strains expressing hyperactive p53 is characterized by perinatal developmental defects, commonly accompanied by early mortality within 2 months of life (**Fig. 4**). For example, mouse strains expressing either of 2 p53 C-terminal truncation mutants, p53Δ31 or p53ΔCTD (p53Δ24), were developed to explore the role of the C-terminal negative regulatory domain of p53.[67,78] As described above, $p53^{\Delta 31/\ \Delta 31}$ mice exhibit lethality between 14 and 43 days along with bone marrow failure and skin hyperpigmentation as well as increased p53 activity and telomere dysfunction.[67] In contrast, $p53^{\Delta CTD/\Delta CTD}$ mice display lethality by 2 weeks after birth and exhibit haematopoietic failure and impaired cerebellar development but only have activated p53 in certain tissues.[78] Another study using $p53^{TSD/-}$ mice explored the role of phosphorylation at threonine (T) 21 and serine (S) 23 by mutating these sites to aspartic acid (D), to mimic p53 activation.[79] These mice exhibit features of premature aging that is correlated with depletion of adult stem cells in various tissues due to increased activation of select p53 target genes, particularly *Puma*.[79] Minimizing the negative regulation of

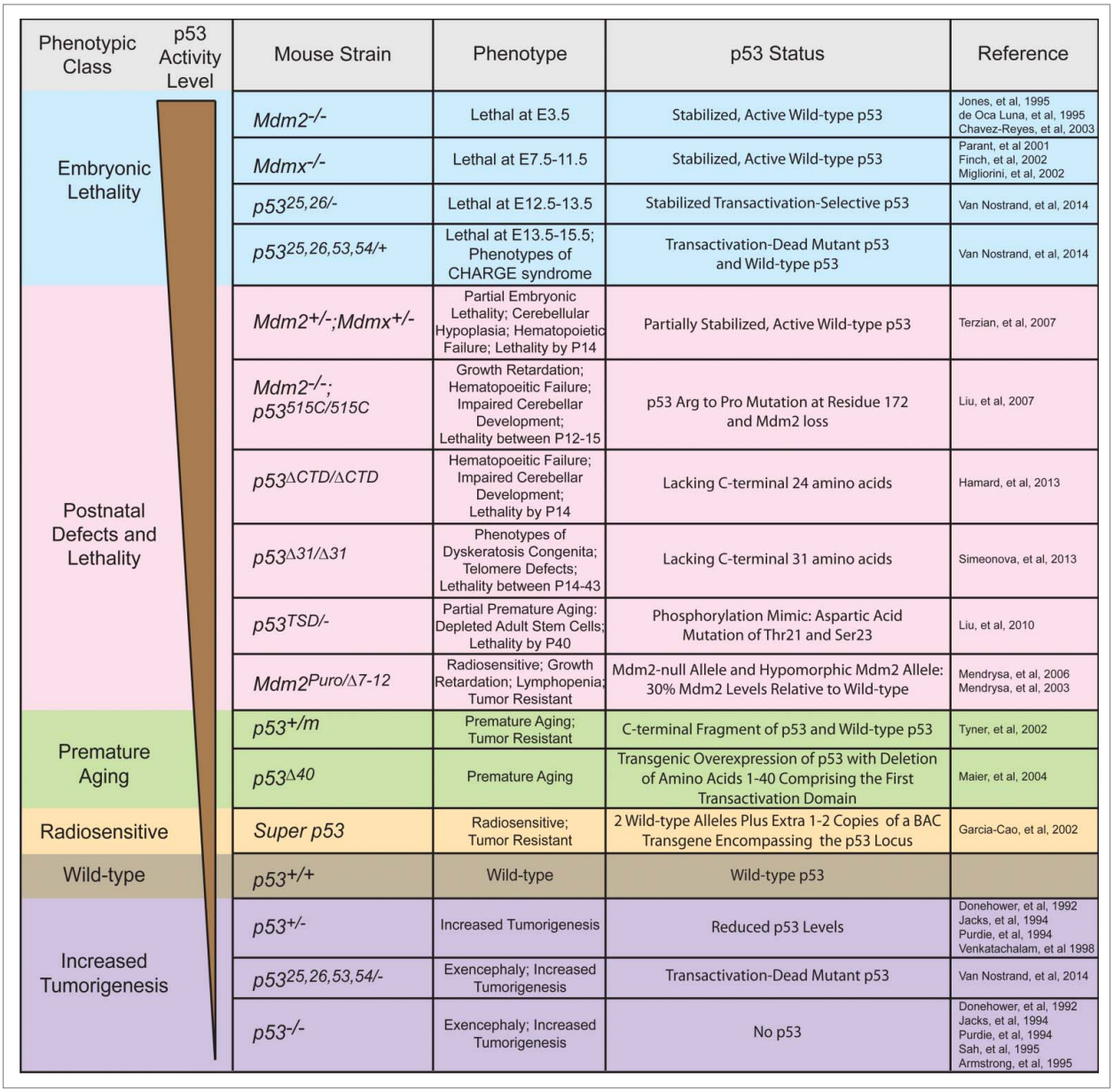

Figure 4. Summary of mouse strains with different levels of p53 activity and associated phenotypes. Mouse strains manifesting varying levels of p53 stabilization and/or activity exhibit diverse phenotypes ranging from embryonic lethality to premature aging to increased tumor susceptibility. The phenotypes and the p53 status in each indicated mouse strain are described.[87-89]

p53 by Mdm2 and Mdmx has provided another approach to examine the consequences of p53 activation on mouse development (**Fig. 4**). *Mdm2;Mdmx* double heterozygous mice display partially penetrant embryonic lethality, associated with exencephaly, as well as perinatal lethality by postnatal day 14 (P14), accompanied by growth retardation, anemia, and hypoplastic tissues.[80] In addition, $Mdm2^{-/-}$ mice homozygous for a p53 hypomorphic mutant lacking apoptotic but not cell-cycle arrest function ($p53^{515C}$) displayed a failure of various progenitor cells to expand, resulting in defective haematopoietic and cerebellar development and death between P12 and P14.[81] In the $Mdm2^{Puro/\Delta 7-12}$ hypomorphic mutant mice, in which one allele is null and the second allele has reduced Mdm2 expression due to the presence of a puromycin cassette in the *Mdm2* locus, the ensuing phenotypes include lymphopenia, growth retardation, and radiosensitivity, associated with enhanced p53 transcriptional activity.[82,83] Interestingly, this strain displays a normal lifespan. Overall, differences in the extent of p53 activation or the spectrum of p53 target genes activated relative to strains displaying embryonic lethality may explain the reduced severity of phenotypes observed in this set of mouse strains.

Other mouse strains expressing hyperactive p53 fail to display developmental defects but ultimately succumb to premature aging (**Fig. 4**). In the *p53 m* mouse

strain, a C-terminal fragment of p53 is produced, provoking premature aging in $p53^{m/+}$ adults.[84] However, unlike the $p53^{25,26,53,54/+}$ embryos, there is no sign of increased basal p53 activity, and instead, stress signals are required to induce the p53 m protein to augment p53 stability and activity. Thus, the cumulative stress with aging may ultimately promote the aging phenotypes. Another mouse strain, overexpressing a truncated, naturally-occurring isoform of p53 that lacks the first transactivation domain (Δ40), also exhibits growth retardation and premature aging.[85] However, expression of Δ40 does not affect wild-type p53 levels, and while some p53 target genes are hyperactivated, a subset of p53 target genes are actually inhibited, which could potentially blunt the effect of the activated p53.[85] In contrast to these mouse strains exhibiting premature aging, the p53 super mice, carrying 1 or 2 transgenes expressing wild-type *p53* on an otherwise wild-type p53 background, age normally and only exhibit enhanced p53 activity in response to DNA damage.[86] The lack of premature aging in the p53 super mice is likely due to the proper negative regulation of p53 protein stability through the N-terminus. Overall, the phenotypic variability observed in these mouse strains expressing hyperactive p53 is likely due to differences in p53 stabilization, p53 transcriptional activity, and the tissues affected in the various mouse models, with the $p53^{25,26,53,54/+}$ embryos exhibiting levels of p53 stability and activity at the higher end of the spectrum.

Future Perspectives and Therapeutic Implications

By unveiling p53 pathway activation as a novel mechanism contributing to the pathogenesis of CHARGE, our work provides significant new insight into the processes triggering CHARGE syndrome. Moreover, we propose that our $p53^{25,26,53,54/+}$ mutant mice represent a new and more penetrant model for human CHARGE syndrome due to the highly overlapping features with human CHARGE. These mice therefore provide a great resource for better understanding the underlying cellular and molecular basis of the disease, including identifying the cell type(s) of origin. Given p53's role as a stress response protein, this new association of p53 with CHARGE syndrome could potentially enhance our understanding of the factors that underlie the great clinical heterogeneity in CHARGE, which could relate at least in part to the ability of p53 to respond to stress signals during pregnancy.[41,42] The identification of a role for p53 in CHARGE syndrome invites investigation of the role of p53 in the genesis of additional developmental syndromes, even beyond those caused by defects in ribosomal biogenesis and neural crest cell function.

The discovery of a causative role for p53 in developmental diseases prompts the question of whether there could be any therapeutic benefit of inhibiting p53. While complete inhibition of p53 during development might be challenging due to the potential for promoting tumorigenesis in either the fetus or the mother, it may be effective to modestly decrease the activity of p53 through mild pharmacological inhibition of p53. Indeed a successful precedent for this strategy was seen with the amelioration of defects in $Tcof1^{+/-}$, $Pax3^{-/-}$, and $Tbx1^{+/-}$ embryos upon administration of the p53-inhibitor Pifithrin-α.[53,73,74] Alternatively, p53 target gene products critical for p53's ability to promote CHARGE phenotypes could be pharmacologically inhibited, which could potentially circumvent the possibility of inadvertently promoting tumorigenesis. Overall, these studies have revealed both the importance of p53 transcriptional activity in triggering developmental phenotypes and a novel role for p53 in CHARGE syndrome, greatly expanding our understanding of the role of p53 in developmental diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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