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Additive effect of TAp63 deficiency on the adrenocortical dysplasia (*acd*) phenotype

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Abstract

Adrenocortical dysplasia (*acd*) is a spontaneous autosomal recessive mouse mutation exhibiting caudal truncation, vertebral segmentation defects, hydronephrosis, limb hypoplasia, and perinatal lethality. *Acd* encodes TPP1, a component of the shelterin complex that maintains telomere integrity, and consequently *acd* mutant mice have telomere dysfunction and genomic instability. We previously showed that apoptosis is the primary mechanism causing the *acd* skeletal phenotype, and that p53 deficiency rescues the skeletal defects of the *acd* phenotype but has no effect on the perinatal lethality. The *Trp63* gene encodes multiple isoforms, which play a role in proliferation, apoptosis, and stem/progenitor cell maintenance. Different p63 isoforms exhibit both proapoptotic (TAp63) and antiapoptotic (DNp63) functions. We hypothesized that deficiency of proapoptotic TAp63 isoforms might rescue the *acd* skeletal phenotype, similar to our previous observations with deficiency of p53. Mice heterozygous for a null allele of TAp63 were crossed to heterozygous acd mice to determine the effect of TA*p63* deficiency on the acd mutant phenotype. In contrast to our results with the *acd* × *p53* cross, skeletal anomalies were not rescued by deficiency of TAp63. In fact, the limb and vertebral anomalies observed in double-mutant embryos were more severe than those of embryos with the *acd* mutation alone, demonstrating a dose-dependent effect. These studies suggest that TAp63 isoforms do not facilitate p53-like apoptosis during development in response to *acd*-mediated telomere dysfunction and are consistent with the proposed roles of TAp63 in maintaining genomic stability.

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Introduction

Adrenocortical dysplasia (*Acd*^{acd}, hereafter denoted *acd*) is an autosomal recessive mouse mutation caused by a splicing defect in the *Acd* gene, which encodes Tpp1, a component of the shelterin telomere complex (Keegan et al.2005). The *acd* mutation results in a RNA splicing defect and is a hypomorphic allele, likely due to residual production of a small amount of appropriately spliced wild-type *Acd* mRNA (Else et al. 2007). Homozygous mutant *acd* mice survive into adulthood on a mixed genetic background with the CAST/Ei strain and have a phenotype consisting of growth retardation, infertility, skin hyperpigmentation, and hypoplastic adrenal glands. Mutant mice do not survive for a significant period of time past birth on the DW/J strain, on which the mutation arose, or on the C57BL6/J (B6) strain (Vlangos et al. 2009). Interestingly, the phenotype of mutant embryos on these latter strains is characterized by caudal truncation with limb hypoplasia, predominantly affecting the lower limbs, and vertebral segmentation defects, resembling caudal regression syndrome in humans (Keegan et al. 2005). Complete deficiency of Acd/Tpp1 results in early embryonic lethality, similar to knockout alleles of other shelterin components (Kibe et al. 2010).

The primary function of Tpp1 is to recruit the single-stranded DNA binding protein(s) Pot1 (a and b) to the telomere and to facilitate binding of Pot1 to the remainder of the shelterin complex and to the single-stranded telomeric DNA overhang (Hockemeyer et al. 2007; Kibe et al. 2010). Tpp1 also physically interacts with the enzymatic component of the telomerase holoenzyme (TERT) and is necessary for recruitment of telomerase to telomeres (Abreu et al. 2010; Wang et al. 2007; Xin et al. 2007). Together, Tpp1 and Pot1 protect chromosome ends from being recognized and processed by the DNA repair machinery as well as recruit telomerase to telomeres. Disruption of Tpp1 and/or Pot1 causes deprotection of the telomeres, resulting in activation of p53 signaling via ATR (Kibe et al. 2010; Rai et al. 2010). Our previous work demonstrated that the skeletal defects observed in *acd* mutant embryos were caused by p53-dependent apoptosis during development (Vlangos et al. 2009). While p53 deficiency did not rescue the perinatal lethality of the *acd* mutation on the B6 background, the vertebral and limb malformations were rescued by p53 deficiency. When *acd* mutant mice on a mixed CAST/Ei background were crossed to p53-deficient mice, a partial phenotypic rescue was observed at the expense of increased numbers of tumors in adult mice (Else et al. 2009).

p53 is a member of a family of transcription factors that encompasses three proteins, p53, p63, and p73 (Yang et al. 2002). p53 was the first family member to be discovered and is widely known for its role in genome surveillance and as a tumor suppressor gene. p63 and p73 have been found to have important roles during development. In particular, p63 is critical for development of mesodermal and ectodermal lineages; mutations in *TRP63* have been identified in individuals with specific ectodermal dysplasias and disorders of clefting, including EEC (ectrodactyly-ectodermal dysplasia-cleft lip/palate), Hay Wells, and limb-mammary syndromes (Brunner et al. 2002). Limb malformations in individuals with these syndromes characteristically include split hand/split foot malformation, defined by central ray reduction defects. Within the p53 family, p53 is the simplest protein, containing only one isoform with a transactivation (TA) domain at the N-terminus and a single C-terminus. Both p63 and p73 contain multiple protein isoforms, with either a TA domain at the N-terminus or a ΔN domain, which lacks transactivation properties. Additionally, both p63 and p73 have at least three different C-termini. Earlier studies showed that p63 TA-domain-containing isoforms exhibit proapoptotic activity, similar to p53, and it is thought that the ΔN isoforms exhibit antiapoptotic activity in a dominant negative manner with respect to p53 (Yang et al. 1998). Both p63 and p73 cooperate with p53 to mediate apoptosis in

response to DNA damage (Flores et al. 2002), and additional studies have suggested that p63 and p73 both play a role as tumor suppressor genes (Flores et al. 2005).

Deletion of the DNA binding domain of the *Trp63* gene in the mouse, and thus knockout of all p63 isoforms, revealed a critical function in the development of epithelial tissues and limbs. Homozygous mutant mice died within 24 h of birth with craniofacial abnormalities, severely truncated limbs, and failure to form a differentiated squamous cell epithelium in the skin. In addition, the progenitor cell population of the basal layer of the epidermis and the apical ectodermal ridge of the limb, where p63 is strongly expressed, were significantly reduced in $p63^{-/-}$ embryos. Based on these findings, it was hypothesized that p63 plays an important role in the maintenance of progenitor cell populations in these tissues (Mills et al. 1999; Yang et al. 1999).

Knockout of the TA-domain-containing (proapoptotic) isoforms of p63 was shown to be important for protecting the female germline from DNA damage, indicating that p63 also plays a role in surveillance of the genome (Suh et al. 2006). These TA-isoform-specific knockout mice were otherwise viable and fertile with a mild phenotype in comparison to mice with knockout of all isoforms of p63. More recent studies of TA-isoform-specific knockout mice have shown that TAp63 is also critical for maintenance of adult skin stem cells (Su et al. 2009). In the absence of TAp63, cells undergo early senescence and exhibit features of premature aging and genomic instability. Development of the skin was normal in these mice, suggesting that the ΔNp63 isoforms are primarily responsible for epithelial development.

Because of the proposed role of TAp63 as a proapoptotic isoform of p63 with properties similar to p53, and given our previous study demonstrating rescue of *acd* mutant skeletal phenotypes but not perinatal lethality by p53 deficiency, we hypothesized that deficiency of TAp63 might also rescue developmental phenotypes associated with the *acd* mutation. In order to test this hypothesis, we crossed *acd* mice to TA-isoform-specific $Trp63^{tm2Fmc}$ (hereafter referred to as *TAp63*)-knockout mice to determine whether TAp63 deficiency could rescue the skeletal defects, similar to what we observed with p53 deficiency, or the perinatal lethality of the *acd* mutation. Our data show that TAp63 deficiency does not rescue the perinatal lethality of the *acd* mutation. Furthermore, the skeletal malformations in *acd* mutant embryos are dramatically exacerbated by deficiency of TAp63, indicating an additive effect. Our results support a role for TAp63 in maintaining genomic stability but not in a "proapoptotic" role in this setting.

Materials and methods

Mice

We have maintained an *acd* breeding colony at the University of Michigan since 2000 when DW/J heterozygous *acd* animals were obtained from cryopreserved stock (stock No. 001595) at the Jackson Laboratory (Bar Harbor, ME, USA) (Keegan et al. 2005). Heterozygous *acd* mice were backcrossed to C57BL6/J mice. The data presented here represent embryos from backcross generation N8. $Trp63^{tm2Fmc}$ mice, maintained on a mixed 129S4/SvJae/Balb/c background (hereafter denoted 129S4/Balb/c), were obtained from Dr. Frank McKeon at Harvard University (Suh et al. 2006). Mice were housed in specific pathogen-free and environmentally controlled conditions with 14-h light/10-h dark cycles. Food and water were provided ad libitum. All experiments involving mice were approved by The University of Michigan University Committee on Use and Care of Animals.

Timed pregnancies

Matings for timed pregnancies were set up using standard animal husbandry techniques. Noon of the day that a vaginal plug was observed was considered E0.5. DNA was isolated from yolk sac as previously described (Keegan et al. 2005; Truett et al. 2000).

Genotyping

Genotyping for the acd mutation was performed on DNA from yolk sac or tail biopsy at 10–15 days of age as previously described (Keegan et al. 2005). Genotyping for the TA*p63*-knockout allele was performed using the primers TAKO4: 5′-ATCTAGCAGCAAGATTAACA A-3′, TAKO5: 5′-GGGAACTTCCTGACTAGGG-3′, and TAKO6: 5′-GCATAGTTCCTACGTTTCAC-3′. TAKO4 and TAKO6 are located just 5′ of exon 2 of the Trp63 gene, and TAKO5 is located within the Neomycin cassette. PCR using an annealing temperature of 61°C and 30 cycles generated a product of 579 bp representing the wild-type allele and a product of 334 bp representing the knockout allele (data not shown).

TA*p63* × *acd* intercross and skeletal analysis

Heterozygous TA*p63*-null mice (mixed 129S4/Balb/c background) were crossed to *acd* heterozygous mice (C57BL/6 background, N8 generation). Resulting double-heterozygous mice (*acd*/+; TAp63$^{+/-}$) were intercrossed to generate double-homozygous mutant mice (*acd/acd*; *TAp63*$^{-/-}$). The number of pups per litter was recorded within 24 h of birth. Genotype distributions at weaning age (p21) and embryonic day (E)15.5–E16.5 were analyzed by χ^2 to determine whether the genotypes were present in expected Mendelian ratios. Embryos were dissected at E15.5–E16.5 in fresh PBS and immediately fixed in 95% ethanol for 4 h. Skeletal staining with Alcian blue and Alizarin red was performed as previously described (Kimmel and Trammell 1981). Stained skeletons were analyzed for morphological abnormalities under a Leica MZ7.5 dissecting microscope. Forelimbs and hindlimbs were designated as normal or abnormal, and specific phenotypes, such as hypoplastic or absent digits, split digits, and absent radii/tibiae, were recorded. The percentage of abnormal/fused vertebrae per embryo was generated by dividing the average number of abnormal/fused vertebrae per embryo by the total number of vertebrae per embryo. Fisher's exact tests (limbs) and unpaired Student's t-tests (vertebrae) were performed to determine statistical significance (www.langsrud.com/fisher.htm).

Results

TA*p63*-null mice exhibit perinatal lethality

To determine whether deficiency of the TAp63 isoform rescues the perinatal lethality of the acd mutation, we first crossed *acd*/+ mice to TA*p63*$^{+/-}$ mice and then analyzed progeny of double-heterozygote intercrosses. Data regarding the number of pups per litter were recorded for 21 of 25 litters (115 of the 132 mice) used in the genotyping analysis. Only two mice were missing from this cohort at the time of ear tag placement at 10–15 days of age. Two additional mice died between ear tag placement and weaning (genotypes *acd/acd*; TA*p63*$^{+/-}$ and +/+; TA*p63*$^{+/-}$). No external malformations, skin lesions, or ulcerations were noted in any of the surviving mice. We found no surviving double-mutant mice at weaning age ($n = 132$, $\chi^2 = 83.29$, df = 8, $p < 0.0005$) (Table 1). However, we observed two *acd/acd*; TA*p63*$^{+/-}$ and four *acd/acd*; TA*p63*$^{+/-}$ mice. These mice were phenotypically similar to surviving acd mutant mice on the CAST/Ei background (data not shown). The TA*p63*-null mice are on a mixed 129S4/Balb/c background, which is likely the reason for the improved survival of the acd mutation compared to the B6 background (CEK, unpublished observations). Of note, we did not observe any surviving TA*p63*$^{-/-}$ mice, although it has

previously been reported that these mice are viable and fertile (Suh et al. 2006). Our data suggest that the TA*p63*-null mice in our cross most likely died in the perinatal period. This finding is also likely due to genetic background effects.

TAp63 deficiency exacerbates the limb malformations in *acd* mutant embryos

To determine whether deficiency of the TAp63 isoform has an effect on the skeletal phenotype of *acd* mutant embryos, we collected embryos at E15.5–E16.5 and analyzed skeletal elements by staining with Alcian blue and Alizarin red. At E15.5-E16.5, we observed Mendelian ratios for the genotype distribution (Table 2). Overall, we observed an increase in the number and severity of limb malformations in both the forelimbs and the hindlimbs of the embryos from this cross (Tables 3, 4, 5) compared to *acd* mutant embryos and embryos from the *acd* × *p53* cross (Keegan et al. 2005; Vlangos et al. 2009). The majority of *acd/acd*; TA$p63^{+/+}$ forelimbs were normal, consistent with previous studies (Keegan et al. 2005) (Fig. 1b). However, none of the *acd/acd*; TA$p63^{-/-}$ forelimbs were normal (Tables 3,5; Fig. 1d). Abnormal phenotypes consisted of hypoplastic or absent digits, missing metacarpals, and absent radii. One embryo (*acd*/+; *TAp63*$^{-/-}$) had a bifid digit; no embryos had polydactyly. In addition, forelimb anomalies were found in a large percentage of *acd*/+; TAp63$^{-/-}$ embryos and acd/acd; TA$p63^{+/-}$ embryos (35% and 54%, respectively), suggesting that the effects of Acd and TAp63 deficiency are additive. However, the forelimb abnormalities in *acd*/+; TAp63$^{-/-}$ embryos were more severe, with absent digits and split-hand malformations. The percentage of abnormal forelimbs increased with increasing numbers of mutant alleles (Table 3). This effect was statistically significant by Fisher's exact test for 2 vs. 3 mutant alleles ($p = 1 \times 10^{-8}$), 2 vs. 4 mutant alleles ($p < 1 \times 10^{-8}$), and 3 vs. 4 mutant alleles ($p < 0.0002$), indicating a dosage-sensitive effect. For the hindlimbs, in general, a higher percentage of abnormalities and more severe abnormalities were observed. None of the hindlimbs in double-mutant embryos were normal (Fig. 1h; Tables 4 and 5). However, several *acd/acd*; TA$p63^{+/+}$ embryos had limb hypoplasia phenotypes, consistent with previous observations (Keegan et al. 2005). Of note, 19 embryos with split-hand or -foot malformations were observed, which has been reported frequently in humans with *TRP63* mutations (Brunner et al. 2002). The majority of embryos (16 of 19) with split-hand/foot malformation had TA$p63^{-/-}$ genotypes. An increase in the percentage of abnormal limbs with an increasing number of mutant alleles was also noted in the hindlimbs, again indicating a dosage-sensitive effect (Table 4). This effect was statistically significant for all comparisons [0 or 1 vs. 2 mutant alleles ($p = 0.004$), 2 vs. 3 mutant alleles ($p = 0.0012$), 2 vs. 4 mutant alleles ($p < 1 \times 10^{-8}$), and 3 vs. 4 mutant alleles ($p = 0.00043$)] by Fisher's exact test.

A comparison of specific limb phenotypes between *acd/acd*; TAp63$^{+/-}$ and *acd/acd*; TA$p63^{-/-}$ embryos also supports an additive effect in both the forelimbs and the hindlimbs (Table 5). The most common forelimb malformation in *acd/acd*; TA$p63^{+/-}$ embryos was a hypoplastic 1st digit, present in 33% of forelimbs, while the majority of *acd/acd*; TAp63$^{-/-}$ embryos had absent digits (81%) and hypoplastic or absent radii (75%). The increased severity in *acd/acd*; TAp63$^{-/-}$ embryos was even more pronounced in the hindlimbs, with 94% of embryos having absent digits and 81% having hypoplastic or absent tibiae.

TAp63 deficiency does not rescue vertebral fusions in *acd* mutant embryos

We previously observed multiple vertebral fusions in *acd/acd* embryos, which were rescued by deficiency of p53 (Vlangos et al. 2009). In the present cross, we observed significant vertebral abnormalities in all *acd/acd* embryos, regardless of TA*p63* genotype (Fig. 2; Table 6). The abnormalities included a significant reduction in the number of tail vertebrae and multiple vertebral fusions. The percentage of abnormal vertebrae per embryo was statistically significant for *acd/acd*; TA$p63^{+/-}$ and *acd/acd*; TA$p63^{-/-}$ embryos vs. wild-

type (+/+; TA*p63*$^{+/+}$) controls by *t*-test (Table 6). While the percent abnormal vertebrae per embryo did not reach statistical significance in *acd/acd*; TA*p63*$^{+/+}$ embryos compared to wild-type controls, this is likely due to low numbers of embryos in both of these groups (Table 6). Although the percentage of abnormal vertebrae per embryo trended higher in *acd/acd*; TAp63$^{-/-}$ embryos compared to *acd/acd*; TA*p63*$^{+/-}$ embryos, it did not reach statistical significance (Fig. 2; Table 6). Vertebral anomalies were observed only in embryos with homozygous acd mutant genotypes, suggesting that TAp63 deficiency alone is not sufficient to cause vertebral anomalies. We also observed a few acd mutant embryos with microphthalmia, rib fusions, and exencephaly, as previously described (Table 7) (Vlangos et al. 2009).

Discussion

Our previous studies showed rescue of *acd* skeletal defects by p53 deficiency and demonstrated that p53-dependent apoptosis is the predominant mechanism leading to skeletal malformations in *acd* mutant embryos (Vlangos et al.2009). However, p53 deficiency did not rescue the perinatal lethality of the *acd* mutation on the C57BL6/J background. Previous studies suggested that the TA isoform of p63 is proapoptotic and plays a role in genome surveillance (Suh et al. 2006; Yang et al. 1998). Based on this information, we hypothesized that deficiency of the TA*p63* isoform might also rescue the skeletal phenotype of *acd* mutant mice, thus supporting a proapoptotic role for p63 during embryonic development. Our results do not support this hypothesis, and, in fact, our data demonstrated overall worsening of the skeletal phenotype in *acd/acd*; TA*p63*$^{-/-}$ embryos. We found a limb phenotype similar to that previously described in *acd/acd*; TA*p63*$^{+/+}$ embryos, primarily affecting the hindlimbs. In contrast, none of the hindlimbs or forelimbs were normal in *acd/acd*; TAp63$^{-/-}$ embryos. In addition, only 57% of the hindlimbs were normal in +/+; TA*p63*$^{-/-}$ embryos, indicating that deficiency of TA*p63* alone is sufficient to cause limb abnormalities. Furthermore, we found a statistically significant difference in the percentage of abnormal hindlimbs and forelimbs with an increasing number of mutant alleles, indicating a dose-dependent effect. We also observed several TAp63-deficient embryos with split-foot malformations, which are commonly observed in humans with *TRP63* mutations (Brunner et al. 2002). Although different syndromic phenotypes caused by *TRP63* mutations tend to cluster in specific functional domains of p63 (Brunner et al.2002), the mutations affecting the limbs in EEC (ectrodactyly-ectodermal dysplasia-cleft lip/palate) syndrome and isolated split-hand/split-foot malformation (SHFM) tend to cluster in the DNA binding domain and would be expected to affect all isoforms of p63.

The vertebral fusions also appeared to be exacerbated by increasing dosage of TAp63 deficiency, although this did not reach statistical significance. However, deficiency of TAp63 alone did not have a significant effect on vertebral formation. This is also consistent with lack of vertebral anomalies in humans with *TRP63* mutations (Brunner et al.2002). Therefore, we conclude from these studies that rescue of the *acd* skeletal phenotype shows specificity for p53, and that the roles of p53 and TAp63 do not overlap during development in the setting of Acd deficiency. While the TA*p63*-knockout mice used in our study, which had been maintained on a mixed 129S4/Balb/c background, were previously reported to be viable and fertile (Suh et al.2006), we did not observe any TA*p63*-knockout mice that survived beyond 24 h of life. This phenotypic difference is likely due to increased contribution of the C57BL/6 background from our cross, which is supported by increased embryonic lethality of TAp63-null mice with increasing B6 contribution in other studies (Su et al. 2009).

Previous studies of p63 have suggested an antiapoptotic role for the ΔN isoform and a proapoptotic role for the TA isoform, and a balance of these opposing isoforms is thought to

be required for normal function (Yang et al.1998). However, a recent study has helped to clarify the role of the TAp63 isoform in the skin (Su et al. 2009). In that study, mice with a complete knockout of TA*p63* had normal development of the skin but developed blisters and skin ulcerations in adulthood, and examination of skin stem and progenitor cells revealed early senescence, evidence of genomic instability with multiple chromosomal aberrations, and increased apoptosis. Deficiency of p53 in the setting of TAp63 deficiency did not rescue the senescence phenotype, yet it did rescue the apoptosis phenotype. TAp63 deficiency also led to early hyperproliferation followed by premature senescence, possibly leading to stem cell exhaustion, as has been observed in other mouse models with telomere dysfunction (Lee et al. 1998; Rudolph et al. 1999). The hyperproliferative phenotype in TA*p63*-deficient mice was thought to be due to inhibition of the cyclin-dependent kinase inhibitor $p57^{kip2}$ (Su et al. 2009). However, other studies have shown that TAp63 functions to induce senescence independently of p53 when transfected into MEF cells (Guo et al. 2009); thus, the role of TAp63 in induction of senescence is not clear. While the exact mechanism by which TAp63 functions to maintain genomic stability is not currently known, expression of TAp63 increases in response to stress, similar to p53 (Su et al. 2009), and it is possible that the role of TAp63 in the maintenance of genomic stability may be related to its role as a transcriptional regulator.

Several aspects of our study support a role for TAp63 in maintaining genomic stability. First, the dose-dependent worsening of the *acd* mutant limb phenotype by TAp63 deficiency suggests that both proteins function in similar or overlapping pathways during limb development. Interestingly, stem cell exhaustion has been proposed as a mechanism in both the TA*p63*-knockout model and in mouse models of telomere dysfunction (Lee et al. 1998; Rudolph et al. 1999; Su et al. 2009). Second, p53 deficiency partially rescues mutant phenotypes in both the *acd* and the TAp63-knockout model (Su et al. 2009; Vlangos et al. 2009). We hypothesize that intact p53, in this model, likely acts to drive cells with genomic instability to undergo apoptosis. However, further studies examining for cellular features of genomic instability and evaluating the effect of p53 deficiency in the current cross will be required to test this hypothesis. In addition, because both Acd and TAp63 are highly expressed in epithelial tissues, it may be interesting to examine the interaction of these proteins in the skin. These studies might be feasible on an alternative genetic background in which both mutant alleles are compatible with survival. Although the *acd* mutant allele is hypomorphic, the study of its characteristic embryonic pheno-type will allow us to answer some of these important questions.

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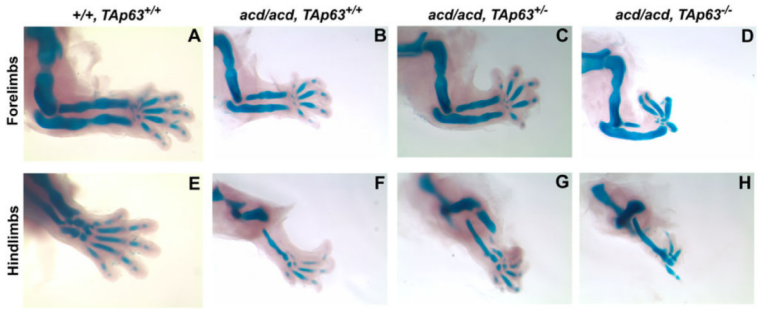

Fig. 1.

TAp63 deficiency significantly exacerbates acd mutant limb phenotype. Skeletal staining of E15.5 embryos from *acd* × TA*p63* intercross. **a, e** Wild-type (+/+; TA$p63^{+/+}$) control showing normal forelimb (**a**) and hindlimb (**e**) phenotypes. **b–d** E15.5 embryo forelimbs. **f–h** E15.5 embryo hindlimbs. *acd/acd*;TA$p63^{+/+}$ (**a, e**) have a phenotype similar to *acd/acd*; $p53^{+/+}$ embryos, with a normal forelimb and absent digits and tibia in the hindlimb. *acd/acd*;TA$p63^{+/-}$ embryos have normal forelimbs (**b**) or minor abnormalities such as a hypoplastic first digit, but an abnormal phenotype in the hindlimbs (**f**) ranging from bifid digits to absent digits and absent or hypoplastic tibiae. *acd/acd*; TA$p63^{-/-}$ embryos (**c, g**) have the most severe phenotype, with missing digits and limb bones in both forelimbs and hindlimbs

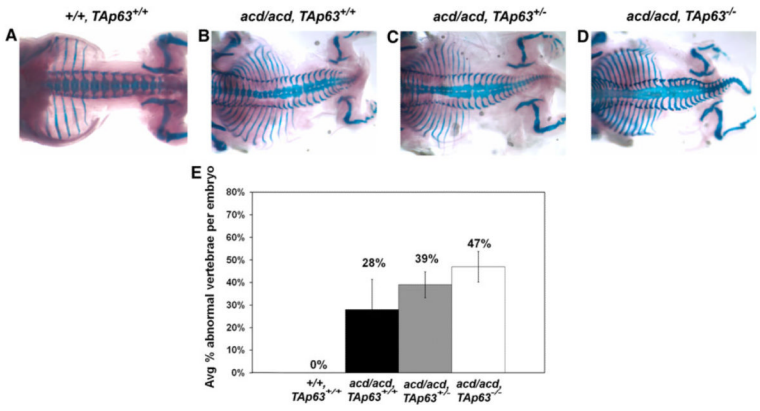

Fig. 2.

TAp63 deficiency exacerbates *acd* mutant vertebral anomalies. Skeletal staining of E15.5 embryos from *acd* × TA*p63* inter-cross. **a** Wild-type (+/+; TA$p63^{+/+}$) control showing normal vertebrae. **b–d** Thoracic, lumbar, and sacral vertebrae of E15.5 embryos showing vertebral fusions in *acd/acd*; TA$p63^{+/+}$, *acd/acd*; TA$p63^{+/-}$, and *acd/acd*; TA$p63^{-/-}$ embryos. **e** Quantification of percent abnormal vertebrae in each embryo. Although not statistically significant, there is a trend toward increasing severity with increasing TAp63 deficiency

Table 1

Genotypes of (*acd*/+ × TA*p63*$^{+/-}$) F_2 progeny at weaning

***Acd* genotype**[a]	**TA*p63* genotype**[b]		
	+/+	**+/−**	**−/−**
+/+	15	28	0
acd/+	30	53	0
acd/acd	2	4	0

$\chi^2 = 83.29$, df = 8, $p < 0.0005$ for predicted Mendelian ratios

[a] *acd* denotes mutant allele

[b] − denotes TA*p63*-null allele

Table 2

Genotypes of (*acd*/+ × TA*p63*$^{+/-}$) F_2 progeny at E15.5

***Acd* genotype**[a]	**TA*p63* genotype**[b]		
	+/+	**+/−**	**−/−**
+/+	3	17	7
acd/+	17	26	10
acd/acd	5	12	8

χ^2 = 5.8, df = 8, 0.70 < p < 0.60 for predicted Mendelian ratios

[a] *acd* denotes mutant allele

[b] − denotes TA*p63*-null allele

Table 3

(*acd*/+ × TA*p63*+/−) intercross embryo forelimb phenotypes[a]

Genotypes (*Acd*; TA*p63*)	No. of forelimbs	No. of mutant alleles[b]	No. (%) abnormal
+/+; +/+	6	0	0 (0%)
+/+; +/−	34	1	0 (0%)
acd/+; +/+	34	1	0 (0%)
+/+; −/−	14	2	0 (0%)
acd/+; +/−	52	2	0 (0%)
acd/acd; +/+	10	2	2 (20%)
acd/+; −/−	20	3	7 (35%)
acd/acd; +/−	24	3	13 (54%)
acd/acd; −/−	16	4	16 (100%)
Total	210		

[a] Numerical values represent number and (percentage) of forelimbs with each phenotype

[b] Statistically significant by Fisher's exact test for 2 vs. 3 mutant alleles ($p = 1 \times 10^{-8}$), 2 vs. 4 mutant alleles ($p < 1 \times 10^{-8}$), and 3 vs. 4 mutant alleles ($p = 0.00018$)

Table 4

(*acd*/+ × TA*p63*$^{+/-}$) intercross embryo hindlimb phenotypes[a]

Genotypes (*Acd*; TA*p63*)	No. of hindlimbs	No. of mutant alleles[b]	No. (%) abnormal
+/+; +/+	6	0	0 (0%)
+/+; +/−	34	1	4 (12%)
acd/+; +/+	34	1	0 (0%)
+/+; −/−	14	2	6 (43%)
acd/+; +/−	52	2	5 (10%)
acd/acd; +/+	10	2	6 (60%)
acd/+; −/−	20	3	6 (30%)
acd/acd; +/−	24	3	17 (71%)
acd/acd; −/−	16	4	16 (100%)
Total	210		

[a] Numerical values represent number and (percentage) of hindlimbs with each phenotype

[b] Statistically significant by Fisher's exact test for 0 or 1 vs. 2 mutant alleles ($p = 0.004$), 2 vs. 3 mutant alleles ($p = 0.0012$), 2 vs. 4 mutant alleles ($p < 1 \times 10^{-8}$), and 3 vs. 4 mutant alleles ($p = 0.00043$)

Table 5

Comparison of *acd/acd*; TA*p63*$^{+/-}$ and *acd/acd*; TA*p63*$^{-/-}$ limb phenotypes[a]

	***acd/acd*; TA*p63*$^{+/-}$ *n* = 24**	***acd/acd*; TA*p63*$^{-/-}$ *n* = 16**
Forelimbs		
Normal	14 (58%)	0 (0%)
Hypoplastic 1st digit	8 (33%)	1 (6%)
Absent digits	2 (8%)	13 (81%)
Split digits	0 (0%)	2 (13%)
Hypoplastic/absent radius	0 (0%)	12 (75%)
Hindlimbs		
Normal	6 (25%)	0 (0%)
Hypoplastic 1st digit	4 (17%)	1 (6%)
Bifid digits	5 (21%)	0 (0%)
Absent digits	8 (33%)	15 (94%)
Split digits	2 (8%)	9 (56%)
Hypoplastic/absent tibia	3 (13%)	13 (81%)

[a]Numerical values represent number and (percentage) of embryos with each phenotype; some embryos exhibited multiple phenotypes

Table 6

Vertebral anomalies in (*acd*/+ × TA*p63*+/−) F_2 progeny

Genotypes (*Acd*; TA*p63*)	No. embryos	Avg No. vertebrae per embryo	Avg No. abnormal vertebrae per embryo	Avg % abnormal vertebrae per embryo[a]
+/+; +/+	3	57.5	0	0
+/+; +/−	17	55.4	2.6	4.7
+/+; −/−	7	56.5	0.9	1.6
acd/+; +/+	17	58.0	0.7	1.2
acd/+; +/−	26	57.1	0.7	1.2
acd/+; −/−	10	56.2	0.9	1.6
acd/acd; +/+	5	46.4	13.2	28.4
acd/acd; +/−	12	51.2	19.9	38.9
acd/acd; −/−	8	51.8	24.1	46.5
Total	105			

[a] Statistically significant difference observed between *acd/acd*; TA*p63*+/− vs. +/+; TAp63+/+ ($p < 0.005$) and *acd/acd*; TA$p63^{-/-}$ vs. +/+; TA$p63^{+/+}$ ($p < 0.005$) by *t*-test. No significant difference between *acd/acd*; TA$p63^{+/+}$ vs. *acd/acd*; TA$p63^{+/-}$ and between *acd/acd*; TA$p63^{+/+}$ vs. *acd/acd*; TA$p63^{-/-}$ by *t*-test

Table 7

(*acd*/+ × TA*p63*$^{+/-}$) intercross embryo phenotypes[a]

Genotypes (*Acd*; TA*p63*)	No. of embryos	Microphthalmia	Fused ribs	Exencephaly
+/+; +/+	3	-	-	-
+/+; +/−	17	-	-	-
+/+; −/−	7	-	-	-
acd/+; +/+	17	-	1	-
acd/+; +/−	26	1	-	-
acd/+; −/−	10	-	-	-
acd/acd; +/+	5	-	1	1
acd/acd; +/−	12	4	-	1
acd/acd;−/−	8	2	2	2
Total	105			

[a]Numerical values represent number of embryos with each phenotype; – indicates 0 embryos with phenotype