Urogenital and caudal dysgenesis in adrenocortical dysplasia (acd) mice is caused by a splicing mutation in a novel telomeric regulator


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Adrenocortical dysplasia (acd) is a spontaneous autosomal recessive mouse mutant with developmental defects in organs derived from the urogenital ridge. In surviving adult mutants, adrenocortical dysplasia and hypofunction are predominant features. Adults are infertile due to lack of mature germ cells, and 50% develop hydronephrosis due to ureteral hyperplasia. We report the identification of a splice donor mutation in a novel gene, which is the mouse ortholog of a newly discovered telomeric regulator. This gene (Acd) has recently been characterized as a novel component of the TRF1 protein complex that controls telomere elongation by telomerase. Characterization of Acd transcripts in mutant animals reveals two abnormal transcripts, consistent with a splicing defect. Expression of a wild-type Acd transgene in acd mutants rescues the observed phenotype. Most mutants die within 1–2 days of life on the original genetic background. Analysis of these mutant embryos reveals variable, yet striking defects in caudal specification, limb patterning and axial skeleton formation. In the tail bud, reduced expression of Wnt3a andDll1 correlates with phenotypic severity of caudal regression. In the limbs, expression of Fgf8 is expanded in the dorsal–ventral axis of the apical ectodermal ridge and shortened in the anterior–posterior axis, consistent with the observed loss of anterior digits in older embryos. The axial skeleton of mutant embryos shows abnormal vertebral fusions in cervical, lumbar and caudal regions. This is the first report to show that a telomeric regulator is required for proper urogenital ridge differentiation, axial skeleton specification and limb patterning in mice.

INTRODUCTION

The adrenocortical dysplasia (acd) mouse was initially described by Beamer et al. (1) in 1994 as a spontaneous, recessively inherited mutation that arose on the DW/J inbred background at the Jackson Laboratory. The phenotypic characteristics of acd mice include reduced survival, poor growth, skin hyperpigmentation and adrenal insufficiency. Hormonal studies in acd mice reveal low corticosterone levels in mutant females and elevated adrenocorticotropic hormone (ACTH) levels in both sexes, consistent with a primary adrenal defect. Histologically, the adrenal glands of acd mice are disorganized with enlarged cortical cells and nuclei. The adrenocortical X zone fails to develop in acd mice, whereas medullary cells are unaffected. In addition to the adrenal phenotype, some acd mutants have hydronephrosis and/or underdeveloped external genitalia. Heterozygotes are unaffected, consistent with autosomal recessive inheritance.

Inherited disorders in humans that result in hypoplasia of the adrenal glands are rare, but they result in significant morbidity in the neonatal period due to severe metabolic disturbances resulting from deficiency of multiple steroid hormones. Though relatively little is known about the genetic etiology of these heterogeneous conditions, adrenal hypoplasia congenita (AHC) has been classified historically into two main categories

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RESULTS

Localization of the acd locus to a 1.5 Mb region on mouse chromosome 8

The acd mutation was previously mapped to mouse chromosome 8 (1). In order to clone the gene responsible for the acd mutation, we created a high resolution genetic map of the acd locus using a classic genetic mapping strategy. Using 145 affected (acd/acd) F2 progeny from an intercross between the mutant strain DW/J-acd/J and CAST/Ei, we mapped the acd mutation to the 51 cM position of mouse chromosome 8, with a maximum interval of 1.7 ± 1.1 cM or ~3.4 Mb (Supplementary Material, Fig. S1).

The public assembly and annotation of the mouse genome (www.ensembl.org) allowed us to identify a bacterial artificial chromosome (BAC) contig covering the acd critical region between markers D8Mit212 and D8Mit12 and to create a physical map of this region. Identification of polymorphisms between DW/J and CAST/Ei on BAC clones RP23-144H18 and RP23-205D5 allowed further narrowing of the acd critical region to ~1.5 Mb (Fig. 1A). Within the 1.5 Mb region, we identified 52 candidate genes for sequence analysis. Candidate genes that had a restricted pattern of expression including adrenal by RT–PCR analysis were prioritized for further study (Supplementary Material, Table S1). Using genomic sequence information from the Ensembl database, PCR primers were designed to amplify known or predicted exons and exon/intron boundaries of these genes for DNA sequence analysis.

The acd mutation is caused by a splicing defect in a novel mouse gene

DNA sequence analysis revealed a single base pair change (G → A) in the +5 position of the splice donor site in the third intervening sequence (IVS3ds +5) of a novel gene (ENSMUSG00000037906) according to Build 30 of the Ensembl database (Fig. 1C and Supplementary Material, Fig. S2). Although all phenotypically affected acd mice were homozygous for this single nucleotide change (Fig. 1C), phenotypically unaffected mice were never homozygous. In addition, the mutation was not present in DNA from unrelated CAST/Ei mice, nor in six other common laboratory strains (data not shown). This gene corresponds to multiple expressed sequence tag (EST) sequences in GenBank (Accession no. BE283096, CK129752 and others). The gene contains eleven exons, spanning approximately three kilobases (kb) of genomic sequence, and has an open reading frame of 1348 basepairs (bp) (Fig. 1B, Supplementary Material, Fig. S2). The transcriptional start site of the mouse gene was mapped to at least 77 bp upstream of the start codon by 5' RACE (Fig. 1B, Supplementary Material, Fig. S3). Sequence analysis of the promoter reveals no consensus TATA box or initiator (Inr) element, but there are 2 CpG islands within 1 kb of the start site.

The mouse protein is predicted to contain 416 amino acids, and it contains no known functional domains. Translated BLAST analysis revealed potential orthologs only in vertebrate species. The N-terminal region of the molecule is well conserved amongst different species (data not shown). The
human ortholog is located on chromosome 16q22.1 with a predicted transcript generated from eleven exons (hypothetical protein 24432). We mapped the transcriptional start site of the human ortholog to a region 66 bp upstream of an ATG that is conserved with the putative initiator ATG codon of the mouse gene. This is in contrast to the predicted transcriptional start site of the human ortholog (hypothetical protein 24432), but consistent with the majority of human EST clones that exhibit significant homology to this transcript (Supplementary Material, Fig. S3). Recently, two groups have independently described the protein product of the human gene (PIP1 or PTOP) as a novel component of the TRF1 complex that controls telomerase-mediated telomere elongation (GenBank accession no. ABY502940) (18,19). We have reserved the gene symbols Acd (mouse) and ACD (human) for this gene through the Mouse Genomic Nomenclature Committee and the HUGO Gene Nomenclature Committee, respectively.

The Acd mutant homozygotes demonstrate aberrant splicing of the Acd gene

To define the molecular consequences of the Acd mutation, we evaluated expression of the Acd gene in acd mutant adrenals and found two abnormal transcripts by RT–PCR and subsequent DNA sequence analysis (Fig. 2A). Using PCR primers in exons 2 and 4, the predicted 240 bp RT–PCR product was observed in wild-type (WT) mice. While acd mutants had an RT–PCR product similar in size to the normal transcript, this PCR product was found by direct sequencing to contain an additional seven base pairs, with splicing occurring at a cryptic splice site beginning 7 bp into the third intron (Fig. 1D). The acd mutants were also found to have a larger 328 bp product, consistent with a cDNA that includes the third intron as a result of loss of splicing due to the mutation. Both WT mice and acd mutants were found to contain a minor splice variant which entirely skips exon 3. This splice product results in alteration of the reading frame and generation of a stop codon in exon 4.

To demonstrate that the abnormal splicing products were present only in acd mutant animals and to determine whether any WT transcripts were present in acd mutants, we designed PCR primers to specifically detect the IVS3-containing (+IVS3), cryptic (CRYPT) and WT splice products (Fig. 2B). As expected, only mutant animals had the +IVS3 and the CRYPT PCR products. No WT transcripts were evident in acd mutant tissues. Both the alternative Acd splice products in acd mutants would be predicted to alter the reading frame of the protein, creating a premature stop codon and a likely non-functional molecule (Fig. 2C). Furthermore, the predicted truncated ACD proteins derived from these splice products in acd mutant mice contain only the N-terminus and would not be expected to interact with POT1 (protection of telomeres-1), as the interaction domain has been mapped to the C-terminus of the protein (18,19).

The Acd gene is expressed in multiple tissues and throughout embryonic development

Expression of the endogenous Acd gene was examined by RT–PCR and northern blot analysis (Fig. 3). Transcripts were present in all eight mouse tissues examined by RT–PCR (Supplementary Material, Table S1). The Acd gene is expressed from birth to adulthood in adrenal glands and in Y1 adrenocortical cells (Fig. 3A). RT–PCR analysis of WT whole embryos from E7 to E18 showed expression of Acd

Figure 1. Physical map of the acd locus on chromosome 8, Acd genomic structure and acd mutation. (A) Schematic diagram showing the 1.5 Mb non-recombinant interval surrounding the acd locus. The positions of the flanking Mit markers D8Mit212 (left) and D8Mit12 (right) and BAC clones RP23-144H18 (left) and RP23-205D5 (right) are shown. The non-recombinant marker D8Mit198 is indicated in the center ~100 kb from the gene. Although there were 52 known or predicted genes in the candidate interval, only high priority genes of known function are shown with their direction of transcription, as noted by the black rectangles above and below the horizontal line. A complete list of the candidate genes is provided in Supplementary Material, Table S1. (B) Genomic structure of the Acd gene. Exons 1–11 are numbered accordingly. The acd mutation at the +5 position of intron 3 is indicated by the asterisk. The start site of the gene as mapped by 5’ RACE is at the beginning of exon 1, and the initiator ATG codon in exon 1 is indicated. PCR primers used for genotyping or RT–PCR are indicated by arrows A–G. 5’mRNA sequencing to contain an additional seven base pairs, with splicing occurring at a cryptic splice site beginning 7 bp into the third intron (Fig. 1D). The cryptic splice donor site is indicated by the horizontal line above the sequence (D).
from the WT transcript. The CRYPT and predicted protein products show the normal 416 amino acid peptide predicted out of frame, respectively, before a stop codon is encountered. To have the first 110 amino acids in frame, followed by 13 and 40 amino acids from the WT transcript because the mutant transcripts are not shown. By northern blot analysis, the band representing the mutant transcript is not significantly different in size throughout development (Fig. 3B). In addition, the Acd gene is expressed in ES cells, but the major transcript observed corresponds to the transcript that skips exon 3. Evaluation of Acd expression by northern blot analysis revealed a major transcript of 1.4 kb in WT (Fig. 3C) and mutant tissues (data not shown). By northern blot analysis, the band representing the mutant transcript is not significantly different in size from the WT transcript because the mutant transcripts are only 7 and 88 bp greater in length. No other significant transcripts were observed (Fig. 3C).

Surviving acd mutants have urogenital defects

When the acd mutation was crossed to the CAST/Ei strain for genetic mapping studies, ~10% of the F1 intercross progeny displayed the mutant phenotype, with many surviving to adulthood. The phenotype of surviving mice is characterized by reduced weight from birth to adulthood (Fig. 7B) and abnormalities of the structures derived from the urogenital ridge—the adrenals, gonads and kidneys. Adrenal glands from acd mutants are small and lack the normal zonation pattern of the adult gland. At high magnification, severe, diffuse adrenocortical dysplasia is evident, accompanied by enlarged, atypical-appearing nuclei with occasional inclusions (Fig. 4A and B). To further explore the relationship between cortical and medullary cells in acd mutants, we performed immunohistochemical analysis with antibodies to 3β-hydroxysteroid dehydrogenase (3βHSD, specific for the adrenal cortex) and tyrosine hydroxylase (TH, adrenal medulla). In mutant adrenal glands, the normal cortical–medullary boundary is absent, and the majority of the gland comprises TH-positive cells (Fig. 4G–J). However, the presence of some 3βHSD-positive cells in the mutant adrenal gland indicates that steroidogenic cells are present, consistent with the fact that corticosterone is detectable in the serum of mutant animals (1).

The external genitalia of acd mutant mice were previously described as underdeveloped. To investigate the gonadal defects in more detail, we evaluated the gonads of acd mutants by standard histochemistry. Indeed, the gonads of acd mutant mice show striking defects. Male acd mutants are infertile, and testis histology shows a reduced number of germ cells in the seminiferous tubules (Fig. 4C and D). Female acd mutants have fewer mature ovarian follicles...
when compared with WT littermates, consistent with their reduced fertility (Fig. 4E and F). We observed hydronephrosis in ~50% of mutant mice, which is associated with smooth muscle hypertrophy/hyperplasia and mucosal hyperplasia/dysplasia of the ureters (data not shown). Many mutants lack fur, and other mutants were noted to have a flaky appearance to the skin. In addition, some mutants have kinked tails. This is consistent with the phenotype previously reported (1), except that polydactyly was never noted in our colony.

**Mutant acd embryos have a caudal dysgenesis phenotype**

In the original description of the acd mutation on the DW/J strain, 40% of the mutants died within 24 h, although some did survive to weaning age (1). Survival improved when the mutant strain was crossed to a number of different genetic backgrounds, including CAST/Ei (1). In the present study, we observed no mutants (0/76 mice) that survived until weaning age on the DW/J background ($\chi^2 = 25.33$, df = 1; $P < 0.001$). At birth, mutant pups are observed, but they die within 24–48 h. This observation, together with the presence of Acd transcripts throughout embryogenesis, prompted us to characterize the embryologic phenotype of acd mutant mice. Analysis of embryonic day 14.5 (E14.5) to E17.5 mutant embryos revealed the presence of variable caudal dysgenesis, demonstrated by reduced formation of caudal structures as well as limb patterning defects (Figs 5 and 6). Gross morphological changes were difficult to observe in acd mutant embryos at E10.5. We therefore chose to examine the expression of markers specific for limb bud and tail bud development by whole mount in situ hybridization at E11.5. We also evaluated skeletal formation in later-stage embryos.

**Axial skeleton defects**

We observed a wide variability in the caudal regression phenotype. The defects ranged from only kinking of the tail (Fig. 5A), to shortening of the tail and posterior axis (Fig. 5B), to dramatic loss of all structures caudal to the hindlimbs (Fig. 5C). The vertebral defects were not limited to the caudal region. In the cervical region, fusions of dorsal arches of the vertebrae were observed (Fig. 5D, arrow). In the lumbar region, there was incomplete midline fusion of the vertebral bodies accompanied by abnormal anterior–posterior fusions (Fig. 5E). In the tail, multiple fusions of caudal (coccygeal) vertebrae were observed (Fig. 5F). Whole mount in situ hybridization at E11.5 revealed reduced expression of Wnt3a and Dll1 but not of Fgf8 in the tip of the developing tail (Fig. 5G–I). The extent of reduction in expression of Wnt3a and Dll1 correlated with the severity of the abnormal morphology of the tail bud. Although this indicates that Acd functions upstream of Wnt3a and Dll1 in the developing tail bud, it remains unknown whether Acd directly or indirectly regulates these genes during embryogenesis.

**Limb defects**

The hindlimb defects were fully penetrant in mutant embryos, with the anterior digits being most severely affected. Abnormalities ranged from a hypoplastic first digit to complete
loss of digits one and two (Fig. 6A–C). In the most severely malformed limbs, zeugopod bones were shortened, with the tibia being more affected (Fig. 6C). At E11.5, Fgf8 staining revealed that the apical ectodermal ridge (AER) was expanded in the dorsal–ventral axis but shortened in the anterior region (Fig. 6D). Even though the overall size of the limb was reduced, expression of sonic hedgehog (Shh) in the zone of polarizing activity (ZPA) was normal (Fig. 6E).

The forelimbs were consistently less affected than the hindlimbs, with digit splaying and rare loss of the most anterior digits (data not shown). However, the forelimb AER revealed a similar expanded expression pattern of Fgf8 (Fig. 6F and G) and normal Shh expression (Fig. 6H).

Expression of an actin-driven Acd transgene rescues the mutant phenotype

To confirm that the mutation in Acd is responsible for the acd mutation. Crosses between these four acd/+ founder animals mated to known acd/+ heterozygous animals generated 7/131 phenotypically affected mice, all of which were negative for the transgene. Eleven phenotypically normal mice were homozygous for the acd mutation, and all were positive for the transgene, demonstrating phenotypic rescue of the acd phenotype (Fig. 7A). All four founder lines produced rescued mutants, and there were no phenotypic differences in the degree of rescue among the different lines. Indeed, phenotypic rescue was observed in every genotypic mutant animal carrying the transgene. Rescued mutants demonstrated appropriate weight gain compared with unaffected (acd/+) transgenic littermates (Fig. 7B). We observed slightly reduced weight in all transgenic mice (acd/+, Tg+) and acd/acd, Tg+) compared with non-transgenic mice. This was not statistically significant after 4 weeks (data not shown). The reason for the reduced weight is unclear, but could possibly be due to increased expression of the Acd gene. In addition, the histological appearance of the adrenal glands of acd mutants containing the transgene showed

Figure 5. Axial skeleton defects in acd homozygous embryos. (A–C) whole embryos displaying various degrees of caudal truncation. (D–F) Alcian blue (cartilage) and alizarin red (bone) staining of vertebrae; arrow indicates fusion of dorsal arches in the cervical vertebrae. Expression of Wnt3a (G) and Dll1 (H), but not Fgf8 (I) is reduced in developing tail bud at E11.5.
the observed phenotype of acd. We have further characterized adrenocortical dysplasia mice were initially reported as a DISCUSSION

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transgenic mice. These data confirm that the locus to a 1.5 Mb region near the 51 cM

caused by disruption of the acd gene. The endogenous Acd gene is widely expressed in adult
tissues and throughout embryonic development, suggesting an important role of the Acd gene during embryogenesis. Indeed, on the DW/J background we uncovered a striking caudal regression and limb malformation phenotype in mutant embryos. The developmental phenotype observed in acd mutants resembles other previously described mouse models of caudal regression, which initially suggested that Acd might participate in these pathways. Specifically, the caudal truncation observed in some acd homozygotes was similar to that observed in Wnt3a−/−Wnt3a−/− or Wnt3a−/−Wnt3a−/− mice (16). However, in Wnt3a-deficient mice, the cervical vertebrae were never affected, and no digital anomalies were described. Mice with a targeted disruption of the Lrp6 gene, which affects global Wnt signaling, have limb and urogenital defects in addition to truncation of the axial skeleton, with loss of posterior hindlimb digits and discontinuous expression of Fgf8 in the hindlimb AER (17). This is in contrast to the hindlimb defects in acd mutant embryos, where loss of anterior digits and a shortened but continuous anterior–posterior Fgf8 expression pattern was observed. These findings suggest that Acd may participate in an additional previously uncharacterized pathway during embryogenesis.

Caudal regression is a complex malformation syndrome in humans with significant genetic and environmental contributions (13), and the exacerbating influence of excess retinoic acid and maternal diabetes is well documented in animal models (14,25). Aside from mutations in HLXB9 in Currarino syndrome (OMIM 176450) (26), no other genes are known to cause this phenotype in humans. Understanding how Acd participates in embryonic patterning will greatly facilitate our understanding of genetic pathways that contribute to caudal regression syndrome.

normal zonation and no cellular or nuclear atypia (Fig. 7C). The testes of rescued mutants showed grossly normal seminiferous tubule structure, and the ovaries had evidence of mature follicular development (Fig. 7D and E). No hydronephrosis or kidney abnormalities were observed in rescued mutant animals (data not shown), and no unusual features were observed in +/+ or acd/+ transgenic mice. These data confirm that the observed phenotype of acd mice is caused by disruption of the Acd gene.

Figure 6. Limb defects in acd homozygous embryos. (A and B) Dorsal view of right hindlimbs, showing digit loss in acd mutants. Digits are numbered 1–3, with 1 being the most medial digit. (C) Skeletal preparation, arrow indicates shortened tibia. (D) The hindlimb AER is broadened and shortened in the anterior region while expression of Shh is normal in the posterior limb (E). (F and G) AER is expanded in the anterior region of the forelimb, while expression of Shh is normal in the posterior area. A, anterior; R, right, L, left.

discerned telomere length regulator referred to as PIP1 or PTOP, a novel component of the TRF1 telomere binding complex in humans, located on chromosome 16q22.1 (18,19). The acd mutation is located at the +5 position of the splice donor consensus site of the third intervening sequence (IVS3ds + 5), changing the +5 G to an A in acd mutant alleles. Two aberrant transcripts are produced in acd mutant animals, resulting in two out-of-frame, premature stop codons that predict truncated protein products. Genotypic mutant mice carrying a WT Acd cDNA transgene grow and develop normally and have no evidence of the adrenal, gonadal or kidney defects observed in acd mice, demonstrating that this mutation is the cause of the acd phenotype.

Splice donor site mutations are well-documented causes of disease in mice and humans (20). In particular, the +5 G residue of the splice donor consensus is highly conserved, and mutation of this base by site-directed mutagenesis has been shown to abrogate splicing by disrupting precursor mRNA-U1 snRNA pairing (21–24). We show that two aberrant transcripts are produced in acd mice, resulting in predicted protein truncations. RT–PCR studies indicate that no WT messenger RNA is observed in acd mutants. However, we cannot exclude the possibility that there are alternative Acd transcripts that might confer some biologic activity, indicating that acd might represent a hypomorphic allele of Acd.

Adrenocortical dysplasia mice were initially reported as a model of human AHC (1). We have further characterized the acd phenotype in adult DW/J × CAST/Ei F2 mice to include aberrant development of the major structures derived from the urogenital ridge. In addition to the adrenocortical defects and hydronephrosis, acd mutant mice have abnormal gonadal development, with complete infertility in males due to decreased numbers of germ cells and reduced fertility in females due to decreased follicular maturation. To identify the molecular basis of the acd mutant phenotype, we mapped the acd locus to a 1.5 Mb region near the 51 CM position of mouse chromosome 8 and identified a candidate gene by sequencing exons in this region. This candidate gene, named Acd, is the mouse ortholog of the recently identified telomere length regulator referred to as PIP1 or PTOP, a novel component of the TRF1 telomere binding complex in humans, located on chromosome 16q22.1 (18,19). The acd mutation is located at the +5 position of the splice donor consensus site of the third intervening sequence (IVS3ds + 5), changing the +5 G to an A in acd mutant alleles. Two aberrant transcripts are produced in acd mutant animals, resulting in two out-of-frame, premature stop codons that predict truncated protein products. Genotypic mutant mice carrying a WT Acd cDNA transgene grow and develop normally and have no evidence of the adrenal, gonadal or kidney defects observed in acd mice, demonstrating that this mutation is the cause of the acd phenotype.

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The contrasting phenotypes of urogenital defects in adult acd mutants on the mixed DW/J CAST/Ei background and more severe embryologic defects on the pure DW/J strain suggest that genetic modifiers play a role in the acd phenotype. Candidate modifier genes might be known or novel components of the telomere binding complex. Other potential candidate modifier genes might be genes that affect telomere length (27), genes that influence activation of p53-mediated apoptosis (28,29) or genes that affect splicing efficiency (30). Indeed, telomeric length varies among mouse strains, with the telomeres in Mus musculus castaneus and Mus spretus being substantially shorter than Mus musculus domesticus substrains (31). The recent identification of Rtel, a novel helicase-like gene, as a genetic modifier responsible for the short telomeres observed in M. spretus (27), supports the hypothesis that genes regulating telomere length may be responsible for modifying the acd phenotype.

Two striking parallels can be drawn between the acd phenotype and in vivo telomerase function. First, defects in acd mice are observed in highly proliferative tissues that exhibit the highest telomerase activity, in both the adult stage (germ cells, skin and hair follicles) and during embryogenesis (limb and tail buds) (32,33). In addition, the postnatal adrenal cortex and testes retain their embryonic expression pattern of the telomerase RNA component longer than all other tissues (33). Secondly, defects in acd mice are reminiscent of the pleiotropic effects of telomerase deficiency in mice. Mice with targeted disruptions of the telomerase RNA component gene (Terc) show infertility and germ cell abnormalities as well as developmental abnormalities, such as open neural tube defects, with successive generations (34,35). Telomerase dysfunction, resulting in genomic instability, classically activates p53-mediated cell-cycle arrest (senescence) and/or apoptosis (36), which might account for the profound embryologic defects in acd mice. The potential role of telomerase dysfunction in human congenital malformation syndromes has not been rigorously explored. However, human syndromes caused by genomic instability, such as Werner syndrome (OMIM 277700), caused by mutations in the RecQ DNA helicase RECQL2, and dyskeratosis congenita (OMIM 127550), caused by mutations in the TERC gene, also share a number of features with adult acd mice, including germ cell failure, adrenal insufficiency and skin abnormalities (37–39).

ACD is proposed to participate in the recruitment of POT1 to single-stranded telomeric DNA and to maintain telomeric structure by inhibition of the telomerase complex (18,19). On the basis of this model, one might predict that acd mice would have telomeric defects. This has not yet been tested. However, mice overexpressing the catalytic component of telomerase (Tert), while showing an increased susceptibility to tumor development, show no developmental defects and no gross change in net telomeric length (40). Taken together,
these observations indicate that (1) *Acd* might have additional functions in development that are independent of telomerase function (41) and (2) *Acd* might participate in other aspects of telomeric function in addition to its role in inhibiting telomerase activity (42). While caudal regression and limb abnormalities can be caused by aberrant retinoic acid and/or Wnt signaling (14,15,43), and retinoic acid has been shown to downregulate telomerase activity in cancer cells (44), the potential interplay between these signaling pathways and telomere maintenance in development has not been explored. The evaluation of telomeric function in *acd* mutant animals will be an important step in defining the role of the *Acd* gene in maintaining telomere stability and will shed insight into the importance of *Acd* in development, aging and cancer.

**MATERIALS AND METHODS**

**Animals**

The *acd* mutation spontaneously arose on the DW/J background at the Jackson Laboratories (Bar Harbor, ME, USA). We obtained DW/J- *acd* heterozygotes (DW/J- *acd/+*) from the cryopreservation unit at the Jackson Laboratories and have maintained a breeding colony at the University of Michigan since 2000. To map the *acd* locus and to generate viable *acd* mutants, DW/J- *acd/+* mice were crossed to CAST/Ei (Jackson Laboratories), and confirmed DW/J- *acd/+* F1 progeny were intercrossed to create *acd/acd* F2 mice. To genotype animals, genomic tail DNA was amplified using primers flanking the mutation: AcdE (5'-ATGCGAAGCGCTTAGCTC-3') and AcdF (5'-TTGTGTGCTGTCTGGAC-3'). All experiments involving animals were performed according to institutionally approved and current animal care guidelines.

**Genetic mapping**

Phenotypically affected F2 intercross mice were genotyped with microsatellite (Mit) markers on mouse chromosome 8. Microsatellite marker information and primer sequences were obtained from Mouse Genome Informatics 3.0 (http://www.informatics.jax.org). Genomic tail DNA was amplified by PCR, and the reactions were run on a 2% NuSieve agarose/1% standard agarose gel and stained with ethidium bromide. Linkage distances were calculated using the Map Manager QTX program.

**Exon sequencing**

Exon-spanning primers were designed using genomic sequence from mouse genome assembly NCBIm30 (Ensembl Genome Assembly, http://mouse30.ensembl.org/Mus_musculus/). PCR was performed using DNA from one affected F2 mouse and one unaffected F2 mouse from the original DW/J- *acd* × CAST/Ei cross. PCR-amplified exons from the mutant animal were purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA, USA) and sequenced by the University of Michigan DNA Sequencing Core. The sequence data were compared with NCBIm30 sequences using Sequencher 4.1.4 software (Gene Codes Corp., Ann Arbor, MI, USA). When a sequence difference was identified, it was verified by amplifying DNA from WT DW/J, CAST/Ei and C57BL6/J control animals.

**RT–PCR**

RNA was extracted from mouse tissues using the RNaseasy kit with optional DNase treatment (Qiagen, Inc.). An aliquot of 1 μg of total RNA was used to produce cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), and 1 μl of the resulting cDNA was used for RT–PCR reactions. Intron-spanning primers were designed using gene sequences available from Ensembl. RT–PCR for *Acd* was performed using either AcdA (5'-ACTTGTGTCAGAACGGAACCC-3') and AcdG reverse primer (5'-GACTGGAGCGCTTGAAGACTC-3') and a forward primer specific for WT transcript (AcdI, 5'-GGGTT-3'), or AcdC (5'-GGGTT-3') or AcdD (5'-TTGTGTGCCTGTCTGGAC-3'). Transcript-specific RT–PCR was performed using the AcdG reverse primer (5'-GACTGGAGCGCTTGAAGACTC-AAAATC-3') and a forward primer specific for WT transcript (AcdI, 5'-GGGTT-3') or AcdD (5'-TTGTGTGCTGTCTGGAC-3'). Transcript-specific RT–PCR was performed using the AcdG reverse primer (5'-GACTGGAGCGCTTGAAGACTC-3') and a forward primer specific for WT transcript (AcdI, 5'-GGGTT-3') or AcdD (5'-TTGTGTGCTGTCTGGAC-3').

**5' RACE**

Primers hRACE-Ia (5'-TGGTACCCAGGAGAACAGA-3'), hRACE-Ib (5'-TTCGCGGCGCAGCAGG-3') and hRACE-II (5'-ACCACTTTTCTCGAGTGAC-3') (human) and mRACE-I (same sequence as AcdB), and mRACE-II (AcdH) (5'-AGCAGACTTGAGAGTGTG-3') were used in 5'-RACE as described in the FirstChoice RLM-RACE kit (Ambion Inc., Austin, TX, USA) using RNA isolated from Y1 (mouse) and NCI-h295R (human) cells.

**Northern blot**

Multiple tissue northern blot was carried out using FirstChoice Northern Blot Mouse Blot I (Ambion Inc.), which is equally loaded with 2 μg poly-A⁺-RNA. The full-length *Acd* cDNA sequence was cut out of a TA-cloning vector (pcR2.1-mAc0) and labeled with 32P-dCTP using the Random Primed DNA Labeling Kit (Roche Applied Sciences, Indianapolis, IN, USA). The blot was incubated overnight (48°C) in hybridization solution containing the probe (ULTRAhyb, Ambion Inc., Austin, TX, USA). Mouse β-actin DECAtemplate (NorthernMax Kit, Ambion Inc.) was used as a control.

**Histology**

For histological analysis, tissues from *acd* mutants and WT littermates were dissected, fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned at 7 μm thickness. Sections were stained with hematoxylin and eosin (H&E) following standard protocols. H&E stained sections were examined under a standard light microscope using Quality One 3.5.8 software (Bio-Rad, Hercules, CA, USA).
Immunohistochemistry

Immunohistochemistry was performed with polyclonal antibodies against βHSD (1:1500; A. Payne, Stanford University Medical Center) and TH (1:500; Pel-Freez Biologicals, Rogers, AR, USA). Biotinylated secondary antibodies were used followed by avidin and biotinylated peroxidase (Vector Laboratories). Antisense mRNA probes for digoxygenin-labeled probe (45) using BM Purple (Roche, CA, USA) to make pCR2.1–6513122 (GenBank accession no. BU516397) by PCR and Acd to make the pCAGGS–mAcd construct, the full-length 1.4 kb cDNA was amplified by the pCR2.1–mAcd. Tissue sections were counterstained with Methyl Green (Vector Laboratories).

Embryos

For timed pregnancies, noon on the day the vaginal plug was observed was considered to be E0.5. Initial developmental studies were carried out on the pure DW/J strain. The acd mutation was subsequently crossed to C57B6/J to put the mutation onto a more well-characterized inbred strain. For these studies, DW/J acd/+ mice were crossed to C57BL6/J +/+ mice (Jackson Laboratories). To determine the severity of the phenotype in the F2 generation, DW/J × C57BL6/J acd/+ F1 progeny were intercrossed. There were no surviving mutants out of 44 progeny, indicating that the phenotypic severity was similar to that on the pure DW/J background. Subsequent timed pregnancies were performed by crossing DW/J × C57BL6/J acd/+ F1 progeny and F2 mutant embryos were analyzed. To genotype embryos, yolk sac DNA was amplified with primers AcdE and AcdF and sequenced by the University of Michigan DNA Sequencing Core.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out with a digoxigenin-labeled probe (45) using BM Purple (Roche, Indianapolis, IN, USA) as the substrate for alkaline phosphatase. Antisense mRNA probes for Fgf8 (46), Shh (47), Dll1 (48) and Wnt3a (49) were prepared as described.

Skeletal staining

Cartilage and bone were stained with Alcian blue and Alizarin Red as previously described (50). Images of cleared skeletons, and whole embryos were captured with a DEI 750 Optronics digital camera and processed using Adobe Photoshop.

Transgenic rescue

To make the pCAGGS–mAcd construct, the full-length 1.4 kb Ac d cDNA was amplified from EST I.M.A.G.E. clone 6513122 (GenBank accession no. BU516397) by PCR and subcloned into the pcR2.1 vector (Invitrogen Corp., Carlsbad, CA, USA) to make pcR2.1–mAcd. The integrity of this clone was verified by DNA sequence analysis. An EcoR1–BamHI fragment from pcR2.1–mAcd containing the full-length cDNA was inserted into the pCAGGS vector (51) at the EcoR1–BglII site just prior to the rabbit β-globin polyA signal. Male DW/J × CAST/Ei acd/+ heterozygotes were mated to either C57BL6/J or DW/J × X CAST/Ei acd/+ females and the fertilized eggs from these matings were injected with the transgene. Mice were genotyped for the transgene using intron-spanning primers AcdA and AcdB, which amplify a 240 bp transgene-specific band and a 490 bp band from genomic DNA as in internal control for DNA quality. Six founder animals were identified, four of which were heterozygous for the acd mutation and were bred for further studies.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online

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