CUL-4A Is Critical for Early Embryonic Development

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Ubiquitin-mediated degradation targets cell cycle regulators for proteolysis. Much of the ubiquitin pathway's substrate specificity is conferred by E3 ubiquitin ligases, and cullins are core components of some E3s. *CUL-4A* encodes one of six mammalian cullins and is amplified and/or overexpressed in breast cancer, which suggests a role in regulating cell cycle progression. To examine *CUL-4A*'s physiologic function, we generated a *CUL-4A* deletion mutation in mice. No viable *CUL-4A*$^{-/-}$ pups and no homozygous mutant embryos as early as 7.5 days postcoitum (dpc) were recovered. However, *CUL-4A*$^{-/-}$ blastocysts are viable, hatch, form an inner cell mass and trophectoderm, and implant (roughly 4.5 dpc), indicating that *CUL-4A*$^{-/-}$ embryos die between 4.5 and 7.5 dpc. Despite 87% similarity between the Cul-4A and Cul-4B cullins, the *CUL-4A*$^{-/-}$ lethal phenotype indicates that *CUL-4A* has one or more distinct function(s). Surprisingly, 44% fewer heterozygous pups were recovered than expected by Mendelian genetics, indicating that many heterozygous embryos also die during gestation due to haploinsufficiency. Taken together, our findings indicate that appropriate *CUL-4A* expression is critical for early embryonic development.

Ubiquitin-mediated degradation plays a critical role in controlling the turnover of cell cycle regulators (reviewed in references 11 and 66). The ATP-dependent attachment of ubiquitin to a ubiquitin-activating enzyme (E1) activates ubiquitin for transfer to a ubiquitin-conjugating enzyme (E2) and then to a ubiquitin ligase (E3), which transfers ubiquitin to a substrate protein (reviewed in references 17 and 46). Repetition of this ubiquitin transferase reaction results in the attachment of a polyubiquitin chain to the substrate, which is then recognized by the 26S proteasome and degraded. Much of the ubiquitin pathway's substrate specificity derives from E3 ligases, so regulating E3 activity is likely to be important for controlling ubiquitin-mediated degradation. Cullins are a core component of a subset of E3 ligases (described below). In this report, we describe in vivo studies that examine the physiologic function of the *CUL-4A* cullin.

Multisubunit RING E3 ligases contain a RING finger protein, an E2, an adaptor protein, a substrate recognition subunit, and a cullin (reviewed in reference 46). An extensively studied type of multisubunit RING E3 is SCF (Skp1, Cdc53/cullin, F-box protein), which was initially characterized for *Saccharomyces cerevisiae* (reviewed in references 11 and 66). Skp1 (the adaptor protein [2]), Cdc53 (the cullin [26, 34, 42, 55, 68]), and Rbx1 (the RING finger protein [3, 23, 52, 56]) make up the SCF core. This core binds various F-box proteins (such as Cdc4, Grr1, and Met30), which are responsible for substrate recognition (reviewed in references 11 and 66). The resulting SCF complexes (SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30}) target for degradation a variety of cell cycle regulators, including Sic1, Far1, Cdc6, Cln1, Cln2, Gic2, and Swe1.

Mammals encode six cullins, Cul-1, Cul-2, Cul-3, Cul-4A, Cul-4B, and Cul-5 (26), and these appear to be components of SCFs or related complexes. For example, Cul-1 is a component of SCF^{Skp2}, which is related to the yeast SCF and is implicated in the ubiquitination of p21, p27, and E2F-1 (6, 30, 32, 33, 40, 60, 64, 72). SCF$^{\beta-TRCP}$ shares the same core components as SCF^{Skp2} and includes the β-TRCP F-box protein, which targets for degradation IκBα and β-catenin (16, 27, 57, 61, 62, 69, 70). An SCF containing Cul-1 and the F-box protein, Cdc4, targets cyclin E for ubiquitin-mediated degradation (36, 59).

Cul-2 is a component of VCB, an E3 whose structure is similar to that of SCF. Along with Cul-2, VCB contains Rbx1, elongin B (a ubiquitin-like protein), and elongin C, which is similar to Skp1 (20, 23, 29, 31, 40, 44). Analogous to Skp1 and F-box proteins, elongin C interacts with SOCS-box proteins, such as elongin A and the von Hippel-Lindau tumor suppressor protein (VHL), and like F-box proteins, these are involved in substrate recognition (1, 12, 24, 25, 58, 73). In response to serum starvation, cells lacking VHL fail to exit the cell cycle and fail to accumulate the cyclin-dependent kinase inhibitor, p27, suggesting a link between p27 stability and an E3 containing VHL (43). This complex also targets a hypoxia-inducible transcription factor, HIF-1α, for degradation by the proteasome (35). In addition, an E3 containing Cul-2, elongin B, and elongin C interacts with another SOCS-box protein, SOCS1, to target the TEL-JAK2 oncoprotein for ubiquitin-mediated degradation (21, 28, 45).

Somewhat less is known about the other mammalian cullins and the complexes they form. Like Cul-1 and Cul-2, Cul-3, Cul-4A, and Cul-5 interact with Rbx1 (40). Cul-3 binds cyclin E and, when overexpressed, stimulates cyclin E ubiquitination (54). Cul-5 is the vasopressin-activated calcium-mobilizing (*VACM-1*) receptor and plays a role in regulating cellular signaling (4, 5). Also, Cul-5 and Rbx1 form complexes with elongins B and C and a SOCS-box protein (elongin A, SOCS1, WSB1, or MUF1), and each immunoprecipitated complex has E3 activity in vitro (22). Recently it was shown that a complex

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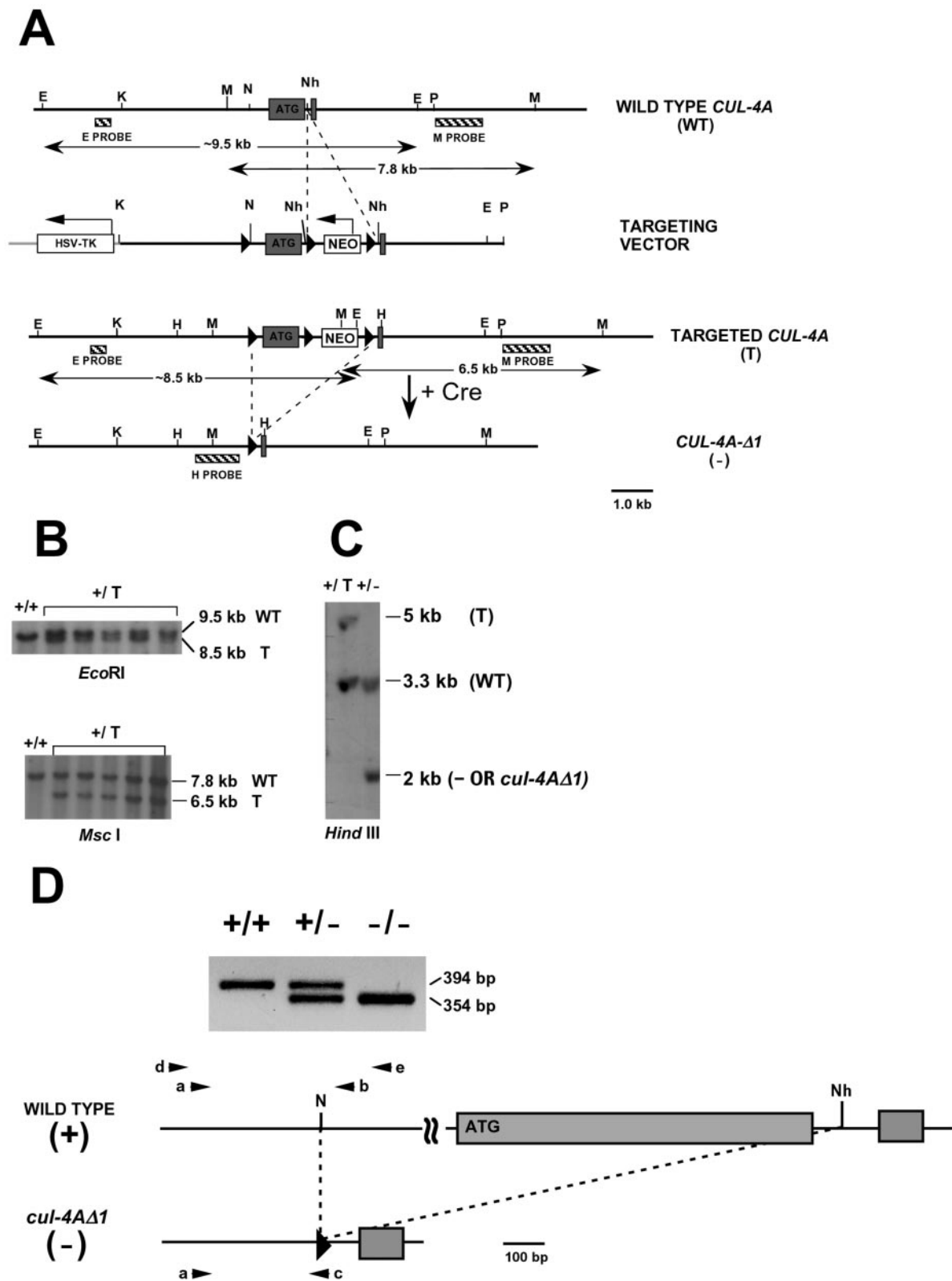
FIG. 1. Targeted disruption of the mouse *CUL-4A* gene. (A) Partial restriction maps of the *CUL-4* locus, targeting vector, targeted locus, and *cul-4AΔ1* deletion. Exons are indicated by black boxes and "ATG" denotes the first coding exon. Restriction enzyme sites are denoted as follows: *Eco*RI, E; *Kpn*I, K; *Msc*I, M; *Not*I, N; *Nhe*I, Nh; *Pst*I, P; *Hin*dIII, H. For the targeting vector, a single *loxP* site (black triangle) and a floxed PGK-neo cassette (black triangles flanking "NEO," neomycin phosphotransferase) were inserted at *Not*I and *Nhe*I sites, respectively, as shown. An HSV-TK (Herpes simplex virus thymidine kinase) cassette lies next to the upstream arm of the *CUL-4A* fragment in the targeting vector. The single-headed arrows show the direction of transcription for the selection markers. In the maps for wild-type and targeted alleles, double-headed arrows underlie

containing Cul-5, elongins B and C, Rbx1, and the adenovirus proteins E4orf6 and E1B55K functions as an E3 to promote p53 ubiquitination and turnover (47).

Thus, cullins and their E3s are involved in the ubiquitin-mediated degradation of cell cycle regulators, and recent findings suggest a similar function for *CUL-4A*. *CUL-4A* mRNA is cell cycle regulated and is high during the G_1- to S-phase transition (9). This gene is amplified and/or overexpressed in 11 breast cancer cell lines and in 31 primary breast cancers, which also suggests a role in regulating cell cycle progression (7). When *CUL-4A* was overexpressed in normal mammary epithelial cells, and after exposure to ionizing radiation, these cells do not accumulate with G_2/M DNA content and do not accumulate p53, indicating that *CUL-4A* does have a cell cycle-regulatory function (15). In addition, *CUL-4A* protein associates with UV-damaged DNA-binding protein (DDB), and *CUL-4A* overexpression was recently shown to promote the ubiquitination and destabilization of the p48 subunit (DDB2) of DDB (8, 38, 53).

In vivo studies demonstrate that cullins are required for normal development. Inactivation of a *Caenorhabditis elegans* cullin, *CUL-1*, causes hyperplasia in all tissues of the developing embryo (26). Mice that are deficient for *CUL-1* fail to develop past 5.5 days postcoitum (dpc) (10, 67), and *CUL-3*-null mice fail to develop past 7.5 dpc (54). However, the role of the *CUL-4A* cullin during development has not been examined.

Here, we report that $CUL-4A^{-/-}$ animals are inviable, and no homozygous mutant embryos were recovered as early as 7.5 dpc. However, $CUL-4A^{-/-}$ blastocysts are viable and are capable of hatching and forming both an inner cell mass and trophectoderm. Approximately 20% of the 7.5-dpc implantation sites appear empty, suggesting that the mutant embryos are able to implant but die between 4.5 and 7.5 dpc. Despite 87% similarity between the Cul-4A and Cul-4B cullins, the $CUL-4A^{-/-}$ lethal phenotype indicates that *CUL-4A* has one or more distinct functions. In addition, fewer heterozygous pups were observed than expected by Mendelian genetics, indicating that many of the $CUL-4A^{+/-}$ embryos also die. Therefore, appropriate *CUL-4A* expression is critical for normal early embryonic development.

MATERIALS AND METHODS

Disruption of *CUL-4A* in embryonic stem cells. To generate a deletion allele of *CUL-4A*, genomic clones were isolated by screening a lambda 129SVJ mouse genomic library (Stratagene, La Jolla, Calif.) with a mouse *CUL-4A* cDNA that includes its entire coding sequence (P. Moeljadi and K. T. Chun, unpublished data). One genomic clone contains approximately 5 kb of 5′ untranslated sequence, the first two coding exons, the intervening intron, and the downstream 11 kb. From this clone, the 3.5-kb *Kpn*I-*Not*I fragment and the downstream 4.4-kb *Not*I-*Pst*I fragment were each subcloned separately into pBS (Stratagene) and used to generate a targeting construct (Fig. 1A). The *Not*I-*Pst*I fragment contains the first two coding exons and the intervening intron. The *Kpn*I site of the

upstream 3.5-kb *Kpn*I-*Not*I fragment was converted with oligonucleotides to an *Eco*RV site, and the resulting *Eco*RV-*Not*I fragment was excised from pBS and inserted into the *Pme*I and *Not*I sites of pTNLOX1-3 (a generous gift from Loren Field, Indiana University School of Medicine). Into the resulting plasmid, a *loxP* site encoded by synthetic oligonucleotides was inserted into the *Not*I site to generate pCUL-4A5′. The inserted oligonucleotides were designed such that the *Not*I site is destroyed at the upstream end of the insert and retained at the downstream end. In the final targeting construct, this *loxP* site lies 0.9 kb upstream of the first coding exon. Next, a 1.7-kb *Not*I-*Asc*I fragment containing *Neo*r transcribed from the *PGK* promoter and flanked by *loxP* sites ("floxed") was excised from pTNLOX1-3, its ends were made blunt with Klenow, and *Nhe*I linkers were attached. This modified fragment was inserted into the 4.4-kb *Not*I-*Pst*I *CUL-4A* fragment in pBS at an *Nhe*I site in the intron between the first and second coding exons and oriented such that *Neo*r was transcribed in the direction opposite to that of *CUL-4A*. The resulting *Not*I-*Pst*I fragment was excised with *Not*I and *Sal*I (pBS polylinker site) and inserted into the *Not*I-*Sal*I sites of pCUL-4A5′ to generate the final targeting construct (Fig. 1A).

Generation of *cul-4AΔ1* mutant mice. Mouse embryonic stem (ES) cell clones hemizygous for *cul-4AΔ1* (Fig. 1A) were injected separately into C57BL/6 blastocysts and implanted into C57BL/6 pseudopregnant females (Jackson Laboratories, Bar Harbor, Maine). From one clone, two highly chimeric males resulted, which were backcrossed to C57BL/6 females, and offspring were genotyped by Southern analysis and PCR (details below) to detect germ line transmission from both males.

Antibody generation and immunoblot analysis. Anti-Cul-4A antiserum was generated in a New Zealand White rabbit (Covance Research Products Inc., Denver, Pa.) using 6-His-tagged Cul-4A (6-His-Cul-4A) immunogen expressed from pET28a-CUL-4AΔN306 in *Escherichia coli* BL21(DE3) (Novagen, Madison, Wisc.). To construct this plasmid, the 2.7-kb *Stu*I-*Not*I fragment containing the coding sequence for the 453 carboxy-terminal amino acids (deleting the first 306 codons, and leaving approximately 60% of the full-length Cul-4A) from the mouse *CUL-4A* cDNA was inserted into the *Nhe*I (made blunt with Klenow) and *Not*I sites of pET28a (Novagen). Protein expression was induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C, and cells were harvested and then lysed with a French press in 50 mM NaH$_2$PO$_4$ (pH 8.0)–300 mM NaCl–10-µg/ml aprotinin–2-µg/ml pepstatin–5-µg/ml leupeptin–100-µg/ml Pefablock. Under these conditions, the 6-His-Cul-4A protein is insoluble, so this fraction was pelleted at 10,000 × *g*, solubilized in protein sample buffer with 2% sodium dodecyl sulfate (SDS), and subjected to SDS-polyacrylamide gel electrophoresis. The 6-His-Cul-4A protein was then electroeluted, concentrated, and injected into rabbits.

For immunoblots, cells were lysed for 30 min on ice in 50 mM Tris-HCl (pH 7.5)–10 mM EDTA–1% SDS–1 mM dithiothreitol–2 mM phenylmethylsulfonyl fluoride–15-µg/ml aprotinin–2-µg/ml pepstatin–5-µg/ml leupeptin followed by probe sonication for 10 s at 4°C. Protein lysates were cleared by centrifugation at 16,000 × *g*, 4°C, for 20 min, and protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, Calif.). 10 µg of total cell lysate were fractionated by SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore; Bedford, Mass.) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0)–10% methanol. Membranes were blocked for 1 h in blocking buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.2% Tween 20, 5% nonfat dry milk), washed three times for 10 min each in KPBS (pH 7.3) (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$) supplemented with 0.2% Tween 20, probed for 1 h with 1/3,000-diluted anti-Cul-4A antiserum in blocking buffer, washed, probed with 1/10,000-diluted anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (A-6154; Sigma-Aldrich Co., St. Louis, Mo.) in blocking buffer, and then washed. Antibodies were visualized by enhanced chemiluminescence (NEN Life Science Products, Boston, Mass.). Actin was detected with antiactin monoclonal antibody (H5441; Sigma-Aldrich Co.) diluted 1/4,000, followed by anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase (NA931; Amersham Life Science; Buckinghamshire, United Kingdom) diluted 1/10,000.

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the distinguishing restriction enzyme fragments identified by Southern analysis. Probes are denoted by hatched boxes. Cre-mediated deletion removed the first coding exon and *Neo*r cassette and generated the *cul-4AΔ1* deletion allele. (B) Southern analyses of targeted ES cell clones digested with either *Eco*RI or *Msc*I. The genotype of each clone is labeled at the top of each autoradiogram. (C) Southern analysis of ES cell clones after Cre-mediated recombination and digestion with *Hin*dIII. The genotype of each clone is labeled at the top of the autoradiogram. (D) Genotyping by PCR. Ethidium bromide-stained gel of PCR products from samples with the corresponding genotypes labeled at the top of the gel. Where the PCR primers, denoted by black triangles, anneal in the wild-type and/or *cul-4AΔ1* alleles is depicted with maps of the corresponding alleles. Note that the scale in these two maps is 10-fold smaller than the maps in panel A.

A deletion mutation in the *CUL-4A* cDNA was generated by PCR and subcloned into pEF-PGKpac for expression in PLB-985 cells, a myelomonoblastic human cell line (65, 71). This mutation removes the first 266 codons and encodes a predicted 59-kDa truncated protein.

Timed pregnancies. To generate timed pregnancies, *CUL-4A*$^{+/-}$ females were injected intraperitoneally with pregnant mare's serum gonadotropin (5 IU per animal; Sigma-Aldrich Co. or Professional Compounding Centers of America, Houston, Tex.), followed by injection with human chorionic gonadotropin (5 IU per animal; Sigma-Aldrich Co. or Serono Laboratories [Randolph, Mass.]) 48 h later and mating overnight with *CUL-4A*$^{+/-}$ males (18). The next morning, males were removed and females were examined for the presence of a vaginal plug. Females were sacrificed at either 7.5, 8.5, or 10.5 dpc to isolate embryos. These were genotyped by PCR (see below).

Isolation of 0.5-dpc embryos and in vitro culturing. Four- to six-week-old heterozygous females were superovulated by treatment with gonadotropins as described above. At 0.5 dpc, females were sacrificed, and embryos were isolated from the oviducts and cultured for 4 days in a drop of M16 medium under mineral oil (18). For blastocyst outgrowths, individual blastocysts were placed on gelatin in 1 ml of ES cell medium (18) supplemented with leukemia inhibitory factor and cultured for 4 more days. Outgrowths were photographed, washed with phospate-buffered saline, and lysed for genotyping by PCR (described below).

Southern and PCR analyses for genotyping. To genotype ES cell lines and identify targeted clones, 10 μg of genomic DNA was digested with *Eco*RI, separated on a 0.5% agarose gel in 1× Tris-borate-EDTA buffer, blotted onto a nylon membrane (Magnagraph; Osmonics, Westborough, Mass.), and probed with a 365-bp PCR fragment (E probe, Fig. 1A) that hybridizes upstream of the targeted region of *CUL-4A*. The 9.5-kb band corresponds to the wild-type allele, and the 8.5-kb band corresponds to the targeted allele (Fig. 1B). To demonstrate further that the targeted alleles are at the *CUL-4A* locus, genomic DNA was digested with *Msc*I, separated by electrophoresis, blotted, and probed with a 1.2-kb *Pst*I-*Eco*RI fragment (M probe; Fig. 1A) that hybridizes downstream of the targeted region of *CUL-4A*. The 7.8-kb fragment corresponds to the wild-type allele, and the 6.5-kb fragment corresponds to the targeted allele (Fig. 1B).

To genotype ES cell lines after Cre-mediated deletion, genomic DNA from each clone was digested with *Hin*dIII, separated by electrophoresis, blotted, and probed with a 1.2-kb PCR fragment that corresponds to the region 2.4 to 1.2 kb upstream of the first coding exon (H probe, Fig. 1A). The 5-kb fragment corresponds to the targeted allele (no deletion), the 3.3-kb fragment corresponds to the wild-type allele, and the 2-kb fragment corresponds to the deletion allele (*cul-4AΔ1*) (Fig. 1C).

To genotype weaned pups, genomic DNA was purified from approximately 0.5 cm of tail and analyzed by either Southern analysis or PCR. For Southern analysis, 10 or 20 μg of DNA were digested with *Hin*dIII and probed with the H probe as described above (Fig. 1A). For PCR analysis, genomic DNA was used as the template in a reaction where wild-type and mutant alleles were detected simultaneously. One oligonucleotide primer (primer A: TGCCAAGAGACAC AAGGTGCTTCG) hybridizes to both wild-type and *cul-4AΔ1* alleles (Fig. 1D). Primer B (CCAACGTGCAGAGGTTACCG) hybridizes to the wild-type allele only, and primer C (GGCCGCATAACTTCGTATAATGTATGCTATACGAA GTTATC) binds to the *loxP* site present only in the *cul-4AΔ1* allele. Briefly, in a 50-μl reaction volume, 100 ng of genomic DNA and 20 pmol each of primers A, B, and C were amplified in PCR buffer (Promega, Madison, Wisc.) supplemented with 1.5 mM MgCl$_2$, 0.2 mM deoxynucleoside triphosphates, and 1.25 U of *Taq* polymerase (Promega) at 95°C for 3 min and then 35 cycles of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C, followed by 6 min at 72°C. Twenty microliters of each reaction mixture was separated on a 2.0% agarose gel in 0.5× Tris-acetate-EDTA buffer. Primers A and B amplify a 394-bp fragment from the wild-type allele, and primers A and C amplify a 354-bp fragment from the *cul-4AΔ1* allele (Fig. 1D).

Embryos at 7.5, 8.5, and 10.5 dpc were genotyped by PCR analysis of DNA purified from either the yolk sac or the whole embryo. Tissues were digested overnight in 300 μl of lysis buffer (100 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 200 μg of proteinase K/ml) at 56°C. DNA was then purified by phenol-chloroform extraction, followed by isopropanol precipitation, and dissolved in 50 μl of 5 mM Tris-HCl (pH 8.0). Two microliters of purified DNA was used for PCR as described above.

Blastocysts and blastocyst outgrowths were genotyped by nested PCR. Outgrowths were washed once with PBS. Then either blastocysts or outgrowths were lysed in 20 μl of PCR lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl$_2$, 0.1 μg of gelatin/ml, 0.45% NP-40, 0.45% Tween 20, 200 μg of proteinase K/ml) at 56°C for 1 h and then 95°C for 15 min. Two microliters were then amplified in the first PCR with primers C (described above), D (CAACAGTG

TTTGCTTGTGCCACTCCCAGGC), and E (TGACAGCTGCCCCCAAAGA AGTGCTCTCAC) (Fig. 1D). The PCR conditions were the same as that described above, except the annealing temperature was 60°C instead of 55°C. Two microliters of the PCR product was used as the template in a subsequent reaction with primers A, B, and C as described for mouse tails and dissected embryos.

RESULTS

Generation and characterization of *CUL-4A*$^{-/-}$ mice. To determine the function of *CUL-4A* during development, mouse ES cell lines with a mutant *CUL-4A* allele were generated. To construct this allele, a 7.9-kb fragment of the *CUL-4A* gene that includes its first two coding exons was isolated and subcloned (Fig. 1A) (see Materials and Methods). Because disrupting either *CUL-1* or *CUL-3* causes an embryonic-lethal phenotype (10, 54, 67), we used the Cre/*loxP* strategy to generate a *CUL-4A* deletion allele (described in this report) as well as a conditional disruption allele (K. T. Chun, unpublished data) (reviewed in reference 48). *LoxP* sites were inserted to flank the first coding exon. The downstream *loxP* site lies in the downstream intron and is followed by a selectable marker for neomycin resistance and another *loxP* site (Fig. 1A). In this way, the first coding exon and approximately 1.1 kb of the upstream sequence is floxed. This entire construct was inserted into a plasmid to generate the targeting construct (Materials and Methods).

Mouse ES cells (CCE916) were transfected with the targeting construct, and G418-resistant, ganciclovir-resistant clones were selected to enrich for homologous recombination events as previously described (49, 50). One hundred twenty-seven of these clones were analyzed by Southern analysis to identify five where the targeting construct had undergone homologous recombination at one of the *CUL-4A* loci (Fig. 1A and B) (see Materials and Methods). Southern analysis with a probe that hybridizes upstream of the targeted region of *CUL-4A* (E probe; Fig. 1A and B) identifies a 9.5-kb fragment for the wild-type allele and an 8.5-kb fragment for the targeted allele. Analysis with a probe that hybridizes downstream of the targeted region (M probe; Fig. 1A and B) identifies 7.8- and 6.5-kb fragments for the wild-type and targeted alleles, respectively. These analyses confirm the presence of the floxed *Neo^r* cassette at the *CUL-4A* locus. To determine whether the *loxP* site upstream of the first coding exon is present, PCR and Southern analyses were performed (data not shown). In one clone this site is absent, but it is present in the other four.

These four clones were transiently transfected with a plasmid that encodes the Cre recombinase (pBS185) (51). This plasmid lacks a selectable marker for mammalian cells, so the Cre recombinase was expressed only transiently in transfected cells. After transfection, cells were plated at low density, individual clones were grown and picked, and 153 were genotyped by Southern analysis (H probe; Fig. 1A and C; also see Materials and Methods) to identify those where the targeted locus (corresponding to a 5-kb fragment) had undergone a Cre-mediated deletion. In 11 clones, the first coding exon and the *Neo^r* marker were deleted (*cul-4AΔ1*, identified by a 2-kb fragment). With one of these cell lines, two chimeric male mice that exhibit germ line transmission of *cul-4AΔ1* were generated (see Materials and Methods).

Upon Cre-mediated recombination to remove the first coding exon, the resulting deletion mutation should result in a

TABLE 1. Genotype of progeny from heterozygous matings[a]

Stage	No. with genotype			No. of Empty implantation sites
	+/+	+/−	−/−	
At weaning	111	124[b]	0[b]	
10.5-dpc embryos	10	15	0[c]	14
8.5-dpc embryos	6	12	0[c]	9
7.5-dpc embryos	12	20	0[c]	8
Blastocysts	10	20	10	

[a] Genotypes were determined by PCR.
[b] Observed frequency departs significantly from the expected ratio of 1:2:1 for +/+:+/−:−/− with a P value of <0.001 (chi-square test).
[c] Observed frequency also departs significantly from the expected ratio with a P value of <0.01 (chi-square test).

nonfunctional *CUL-4A* gene. This deletion removes the first 49 codons and the first base pair of the 50th, generating a frame-shift mutation as well. Two out-of-frame ATG codons are present before the first in-frame ATG occurs at codon 146. Therefore, even in the unlikely event that translation begins at codon 146, the first 145 amino acids would be absent, resulting in a truncated protein that is only 614 amino acids long instead of the wild-type 759 amino acids. The first 108 amino acids of Cul-2, the first 249 amino acids of Cul-1, and the first 280 amino acids of Cdc53 are required for these cullins to function normally (10, 54, 67). Therefore, it was likely that deleting the first coding exon of *CUL-4A* would result in a null phenotype.

Offspring hemizygous for the *cul-4AΔ1* allele were inter-crossed, and 235 weaned pups were genotyped by PCR analysis (Fig. 1A and D; Table 1; also see Materials and Methods). None were homozygous for the deletion allele, indicating that animals lacking functional *CUL-4A* are not viable (statistically significant at $P < 0.001$, chi-square test). Surprisingly, 47% of the viable pups were wild type and only 53% were heterozygotes. Assuming a Mendelian ratio of 1:2 for wild-type animals to heterozygotes, twice the number of wild-type animals (111) would be expected to be heterozygotes (222). Therefore, approximately 44% of these expected heterozygotes are also inviable (222 heterozygotes expected minus 124 observed, out of 222 heterozygotes expected), and this deviation from the expected is statistically significant ($P < 0.001$, chi-square test). However, for the viable heterozygotes, there is no overt phenotypic difference between these and wild-type animals.

As described above, deletion of the first coding exon left an in-frame ATG at codon 146, so it was possible that a truncated, 614-amino-acid (approximately 68-kDa) *CUL-4A* protein was expressed instead of the 84-kDa wild-type protein. To examine whether the *cul-4AΔ1* allele is null or results in the expression of a truncated *CUL-4A* protein, thymus total cell lysates from wild type and hemizygotes were analyzed by immunoblot and probed with anti-Cul-4A antiserum (Materials and Methods). As shown in Fig. 2, no truncated forms of Cul-4A were detected. In addition, the amount of Cul-4A expressed in the hemizygote is roughly half that expressed in the wild type. If a truncated gene product had been expressed, this antiserum should have detected it, because all of the portion of Cul-4A used as the immunogen is included in the possible truncated product from the *cul-4AΔ1* allele. To confirm that the antiserum would detect this possible *cul-4AΔ1* truncated protein, a truncated Cul-4A protein that lacks the first 266 amino acids
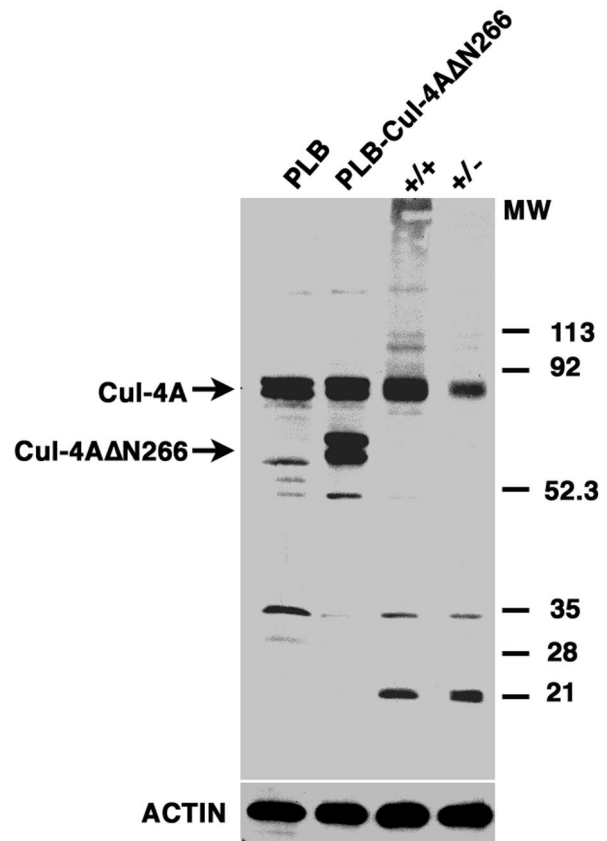

FIG. 2. A truncated protein is not expressed from *cul-4AΔ1*. Protein lysates were prepared from wild-type PLB-985 cells (PLB), PLB-985 cells expressing a truncated *CUL-4A* protein that lacks its amino-terminal 266 amino acids (PLB-Cul-4AΔN266), thymus total lysate from a wild-type mouse (+/+), and thymus total lysate from a hemizygous littermate (+/−) (see Materials and Methods). Lysates were fractionated on a 7.5% polyacrylamide gel, and an immunoblot was probed with anti-Cul-4A antiserum. Full-length and truncated Cul-4A are each denoted by an arrow, and actin levels were measured to ensure equal loading. MW, molecular weight, given in thousands.

was expressed in PLB-985 cells (a human cell line; see Materials and Methods). As shown in Fig. 2, the anti-Cul-4A antiserum clearly recognizes this truncated protein. Endogenous, full-length Cul-4A and truncated Cul-4A each appear as doublets, which is consistent with the finding that Cul-4A is modified by the attachment of a ubiquitin-related peptide, Nedd8 (19, 41). Therefore, it is likely that no truncated products are expressed and that *cul-4AΔ1* is a null allele.

To determine at what stage of development the homozygous mutant embryos die, 7.5-, 8.5-, and 10.5-dpc embryos from heterozygote intercrosses were isolated and genotyped by PCR (Table 1; see Materials and Methods). No homozygous mutant embryos were recovered at each stage, indicating that *CUL-4A⁻/⁻* embryos die prior to 7.5 dpc ($P < 0.01$). Approximately 20% (8 of 40) of the 7.5-dpc implantation sites appeared empty, half (20 of 40) yielded heterozygous embryos, and similar proportions were observed at 8.5 dpc. However, a slightly smaller proportion than the expected (15 of 39, or 38% instead of 50%) yielded heterozygotes at 10.5 dpc, but this deviation is not statistically significant.
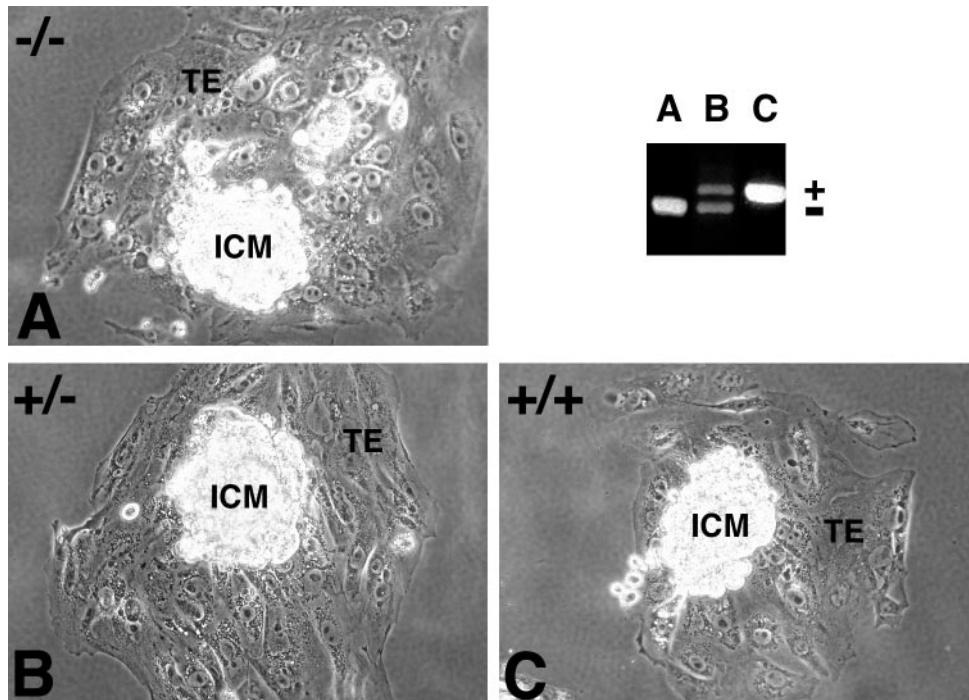
FIG. 3. CUL-4A$^{-/-}$ blastocysts grown in vitro exhibit normal outgrowth characteristics. Blastocysts derived from CUL-4A$^{+/-}$ intercrosses were cultured in vitro for 4 days. The presence of both an inner cell mass (ICM) and trophectoderm (TE) were detected in each outgrowth. The lens objective was ×32. The genotype of each outgrowth was determined by PCR and appears in the upper left corner of each panel. The electrophoresed PCR products for genotyping appear with each lane labeled with the letter of the corresponding outgrowth. PCR products corresponding to the wild-type or deletion allele are labeled by + or −, respectively (see Materials and Methods).

In vitro analysis of early embryos. The failure of CUL-4A$^{-/-}$ embryos to develop past 7.5 dpc suggests that CUL-4A may be required for cell viability. To begin to assess CUL-4A's role in this function, 0.5-dpc embryos from heterozygote intercrosses were isolated and allowed to develop for 4 days in vitro, and then viable blastocysts were genotyped by PCR (see Materials and Methods). Twenty-five percent of these were homozygous mutant, 50% were heterozygotes, and 25% were wild type, indicating that CUL-4A is not required for blastocyst formation (Table 1). Similarly prepared blastocysts were cultured on gelatinized petri dishes 4 days longer (in the presence of LIF) and then genotyped. Mutant blastocysts formed an inner cell mass and trophectoderm and were morphologically indistinguishable from wild-type and heterozygous outgrowths (Fig. 3).

DISCUSSION

To determine the in vivo function of CUL-4A, we generated a mouse line hemizygous for a CUL-4A deletion allele. No viable, homozygous mutant offspring were obtained from heterozygote intercrosses, indicating that CUL-4A is required for normal embryonic development. One quarter of the viable blastocysts (roughly 3.5-dpc embryos) recovered from similar intercrosses were homozygous mutant, and such embryos were able to hatch and form an inner cell mass and trophectoderm, indicating that CUL-4A is not required for development to this stage. However, upon analysis of 7.5- to 10.5-dpc embryos, no CUL-4A$^{-/-}$ embryos were recovered. Approximately one-

quarter of the implantation sites observed at these time points appeared empty, indicating that CUL-4A$^{-/-}$ embryos are able to hatch and implant (4.5 dpc) but fail to develop past a point between this stage and 7.5 dpc.

Surprisingly, 44% fewer heterozygous pups were observed than expected by Mendelian genetics, indicating that many of the heterozygotes from these intercrosses are also dying and suggesting that appropriate CUL-4A expression is required for embryonic development. No truncated CUL-4A gene product was detected in lysates from heterozygotes, and the amount of full-length Cul-4A was roughly half that in wild-type littermates, so it is likely that this semidominance results from a reduction in CUL-4A expression and not from a dominant mutant, truncated Cul-4A. Also, the Mendelian proportion of heterozygotes was observed at 10.5 dpc, indicating that the inviable heterozygotes are dying at a stage later than the homozygous mutants. None of the other cullin null alleles generated thus far cause an apparent phenotype in heterozygotes, so the haploinsufficiency of the CUL-4A null allele is novel for this gene family.

Other cullins participate in several different E3 complexes, and each complex often has multiple substrates (reviewed in references 11 and 66). Therefore, it is likely that there are multiple substrates for the Cul-4A-containing E3(s) and that the failure to regulate the degradation of one or more of these substrates results in early embryonic death. A candidate for such a factor is DDB2, which was identified as a likely substrate of Cul-4A-containing E3 (8, 38, 53). DDB2 is mutated in some xeroderma pigmentosum group E individuals, and this gene is

required for the p53-dependent activation of the global genomic DNA repair of UV radiation damage (39, 63). A database search for *DDB2* cDNAs identified an expressed sequence tag (GenBank accession no. AA516636) from a cDNA that was generated from 6.5-dpc mouse embryos, so it is likely that *DDB2* is expressed during the same stage of development when *CUL-4A* is required. Therefore, dysregulated DDB2 degradation may contribute to the embryonic-lethal phenotype of *CUL-4A*$^{-/-}$ mice. However, there is no other evidence to indicate a requirement for *DDB2* during development. For example, it is not known whether the overexpression of *DDB2* by another means (e.g., *DDB2* transgenic mice) results in the same phenotype. The *cul-4AΔ1* mice provide a suitable system with which to elucidate the molecular mechanism of *CUL-4A* function during early embryonic development.

As components of ubiquitin ligases that regulate cell cycle regulators, cullins have been shown to be critical for normal cell cycle progression, and this appears to be the case for *CUL-4A* as well. The observations that *CUL-4A* is overexpressed in breast cancer and that its mRNA levels are cell cycle regulated suggest a role in regulating cell cycle progression (7, 9). Also, *CUL-4A* overexpression appears to interfere with the G$_2$/M checkpoint after exposure to ionizing radiation in mammary epithelial cells (15). At 6.5 dpc, there are approximately 500 cells in the mouse embryo, which rapidly increase to over 100,000 cells in the 8.5-dpc embryo, so it is likely that during this rapid proliferation, the proper regulation of cell cycle progression is critical. Our results indicate that *CUL-4A* is required during roughly this same period of development. Also, *CUL-1* and *CUL-3* are each required during this period of embryonic development, and each is involved in targeting cell cycle regulators for degradation (10, 54, 67). Apparently, multiple E3s, including one containing Cul-4A, are required to help orchestrate developmental events occurring during this early embryonic stage.

The Cul-4A amino acid sequence is 78% identical and 87% similar to that encoded by its most closely related gene, *CUL-4B* (GenBank accession no. NM_028288). However, despite this high degree of similarity, disrupting *CUL-4A* causes a dramatic phenotype, demonstrating that one or more of *CUL-4A*'s functions are distinct from those of *CUL-4B*. In fact, the predicted amino-terminal amino acid sequence for Cul-4B is different from the first 40 amino acids predicted for Cul-4A, while the remainder of Cul-4A and -4B are 82% identical and 91% similar. In other cullins, the amino terminus is required for binding Skp1 or elongin C and F-box or SOCS-box proteins, which are all involved in substrate recognition (11). Perhaps Cul-4A and Cul-4B bind different adaptor and substrate recognition subunits, which results in their corresponding E3s targeting different substrates for degradation and, in turn, distinct functions. Alternatively, these two genes might exhibit very different expression patterns, resulting in their requirement during different periods of development and/or in different tissues. In fact, we and others observed that in adult human tissues, *CUL-4A* is most highly expressed in skeletal muscle, cardiac muscle, and the testis (A. W. Roberts and K. T. Chun, unpublished data; 19). *CUL-4B* is most highly expressed in the spleen, prostate, testis, ovary, and colon and in leukocytes (19). Also, *CUL-4B* expression was reported to be especially high at

day 11 of mouse embryonic development, while *CUL-4A* expression was not markedly higher at this stage (19).

Studies with mice, *C. elegans*, plants, and *Dictyostelium* suggest that cullins, their E3 ligases, and ubiquitin-mediated degradation play critical roles in development (10, 13, 14, 26, 37, 54, 67). For example, the plant hormone auxin is critical for regulating cell division and differentiation, and in *Arabidopsis thaliana*, mutations in genes encoding SCF subunits (ASK1, related to Skp1, and TIR1, an F-box protein) dramatically alter developmental responses to auxin (14). In *Dictyostelium*, an apparent E3 containing CulA and FbxA target RegA (required for normal development) for degradation (37). A mutation in either *CulA* (most closely related to mouse and human *CUL-1*) or *FbxA* (encoding an F-box protein) causes a failure to downregulate RegA and defects in cell-type differentiation and development.

The studies described here demonstrate that *CUL-4A* contributes an essential function for early mammalian development and suggest that as little as a twofold decrease in expression can cause embryonic death. Nearly half of mice heterozygous for the *cul-4AΔ1* allele die in utero, apparently during the second half of gestation, thus providing a useful system with which to study the role of *CUL-4A* in later stages of development. Further study will also be required to determine the mechanism by which *CUL-4A* is required for early embryonic development. This will include comparing differentiation and cell cycle progression during early embryonic development in *CUL-4A*-null and wild-type animals. The early death of *CUL-4A*$^{-/-}$ embryos makes discerning these processes difficult. We are currently generating *CUL-4A*$^{-/-}$ ES cell lines to examine whether normal differentiation in vitro requires *CUL-4A* and if so, to identify Cul-4A downstream effectors, perhaps E3 substrates, whose dysregulation ultimately leads to the embryonic-lethal phenotype.

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