**L2dtl Is Essential for Cell Survival and Nuclear Division in Early Mouse Embryonic Development**

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*(lethal (2) denticleless), is an embryonic lethal homozygous mutation initially identified in *Drosophila melanogaster* that produces embryos that lack ventral denticle belts. In addition to nucleotide sequence, bioinformatic analysis has revealed a conservation of critical functional motifs among the human L2DTL, mouse L2dtl, and *Drosophila* l(2)dtl proteins. The function of the L2DTL protein in the development of mammalian embryos was studied using targeted disruption of the L2dtl gene in mice. The knock-out resulted in early embryonic lethality. L2dtl−/− embryos were deformed and terminated development at the 4–8-cell stage. Microinjection of a small interfering RNA (siRNA) vector (siRNA-L2dtl) into the two-cell stage nuclei of wild-type mouse embryos led to cell cycle progression failure, termination of cell division, and, eventually, embryonic death during the preimplantation stage. Morphological studies of the embryos 54 h after injection showed fragmentation of mitotic chromosomes and chromosomal lagging, hallmarks of mitotic catastrophe. The siRNA-L2dtl-treated embryos eventually lysed and failed to develop into blastocysts after 72 h of *in vitro* culturing. However, the embryos developed normally after they were microinjected into one nucleus of the two-celled embryos. The siRNA studies in HeLa cells showed that L2dtl protein depletion results in multinucleation and down-regulation of phosphatidylinositol 3-kinase, proliferating cell nuclear antigen, and PTTG1/securin, which might partially explain the mitotic catastrophe observed in L2dtl-depleted mouse embryos. Based on these findings, we conclude that L2dtl gene expression is essential for very early mouse embryonic development.

The l(2)dtl (lethal (2) denticless) gene in *Drosophila melanogaster* encodes a unique 83-kDa protein that is up-regulated under heat shock conditions (1). Accordingly, *Drosophila* l(2)dtl shares common features with HSP90 family members, including protein expression pattern, structure of the promoter region, and mRNA expression throughout development in a wild-type *Dm* strain (1). Homozygous mutations of l(2)dtl result in the absence of ventral denticle belts and embryonic lethality in *Drosophila*, hence the name of this gene (1). L2DTL (GenBank™ accession number AF195765), the human ortholog of *Drosophila* l(2)dtl, was recently shown to encode a nuclear matrix-associated protein that is down-regulated during the retinoic acid-induced neuronal differentiation of NT2 cells. Hence, human L2DTL is also known as ramp (retinoid acid-regulated nuclear matrix-associated protein) (2).

In humans, L2DTL is more highly expressed in the fetal tissues of the brain, lung, liver, and kidney than in normal adult tissues, suggesting a specific role during embryogenesis (2). Initial characterization of L2DTL protein in NT2 cells shows that it is an active participant in cell proliferation (2), as was hypothesized for other WD40 repeat-containing proteins (3–7). Using differential display (8, 9) to screen aberrant gene expression patterns revealed frequent L2DTL overexpression in hepatocellular carcinoma (HCC) cells (10–13). Overexpression of L2DTL mRNA in unifocal primary HCC is now associated with larger tumor size, high grade, and high stage HCC and poorer survival, highlighting the potential role of L2DTL in HCC progression. Despite these observations, the physiological function of L2DTL, particularly its role in embryogenesis, has not been resolved. Our bioinformatic analysis5 showed that the amino acid sequence of human L2DTL is 89% homologous to mouse L2dtl (GenBank™ accession number NM-029766) and 25% homologous to *Drosophila* l(2)dtl (GenBank™ accession number X83414). Although the human and mouse genes have a unique sequence at the carboxyl terminus that is not present in the *Drosophila* counterpart, all of the other major functional motifs, such as WD repeats, a nuclear localization signal, a D-box, a KEN-box, and PEST sequences, are conserved.

The goal of this study was to investigate the physiological roles of L2dtl, an apparent ortholog of l(2)dtl, in embryonic mouse development. Homologous recombination in mouse

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**Notes:**

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3. The abbreviations used are: HCC, hepatocellular carcinoma; siRNA, small interfering RNA; RT, reverse transcription; dpc, days postcoitum; En, embryonic day n; RT, reverse transcription; DAPI, 4′-6-diamidino-2-phenylindole; RNAi, RNA interference; KO, knockout.

4. H.-W. Pan, H.-Y. Chou, S.-H. Liu, S.-Y. Peng, C.-L. Liu, and H.-C. Hsu (2006) *Cell Cycle*, in press.

5. C.-L. Liu, I.-S. Yu, H.-W. Pan, S.-W. Lin, and H.-C. Hsu, unpublished results.
Embryonic Lethality in L2dtl Knock-out Mice

embryonic stem (ES) cells was used to generate L2dtl knock-out mice. Initial breeding and characterization revealed the embryonic lethality of L2dtl null mice. The development of L2dtl null embryos ceased at the 4–8-cell stage. A similar phenomenon was observed in two-cell stage embryos using siRNA knockdown of the L2dtl transcript. The results indicate that L2dtl is required for early embryonic development in mice.

MATERIALS AND METHODS

Computer Analysis of the Mouse L2dtl Gene—The genomic sequence of L2dtl was obtained from strain 129 in the Celera mouse data base. The sequence revealed a possible L2dtl gene with several partially unidentified sequences and ambiguous exon-intron boundaries. The sequence gaps within possible exons were completed by sequencing the 2.4-kb full-length cDNA, which was obtained by reverse transcription-polymerase chain reaction (RT-PCR) of RNA extracted from fetal liver extracts. The identity of the 2.4-kb sequences was confirmed by comparing the sequences to the GenBank™ mouse L2dtl reference (accession number NM-029766). The 2.4-kb sequence was further analyzed using ELM (available on the World Wide Web at elm.eu.org), SMART (available on the World Wide Web at smart.embl-heidelberg.de/), PSORT (available on the World Wide Web at www.psort.org/), and PESTfind (available on the World Wide Web at www.psort.org/). DEDON software programs were used to deduce amino acid sequence and functional domains.

Semi-quantitative RT-PCR Measurements—Semi-quantitative RT-PCR was performed to detect relative RNA quantities as described previously (14). Total RNA was prepared using TRIzol reagent (Invitrogen). After treatment with DNase I, total RNA was transcribed with Superscript II (Invitrogen) using oligo(dT) primers. The primer pairs were 5′-AACATT-ATGATCTGGGACACCAGG-3′ and 5′-ATCACCTGGTTC-CTTCTCTAGG-3′ for L2dtl and 5′-GACCACAGTTCCATGCTC- CATCAC-3′ and 5′-TCCACCACCCCTGTGCCTGTA-3′ for Gapdh. For each gene, the number of PCR cycles was optimized to detect differences in mRNA amounts: 30 cycles for Gapdh and 35 cycles for L2dtl. Each cycle consisted of 30 s at 95 °C, 40 s at 55 °C, and 1 min at 72 °C using Taq polymerase (BerTag, DCB, Taipei, Taiwan). PCR products were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide.

Disruption of L2DTL in Mouse Embryonic Stem Cells—A 10-kb L2dtl genomic DNA fragment coding for exons 1–5 was isolated from a BAC clone harboring 100 kb of 129/Sv genomic DNA (high density mouse BAC colony membrane, ResGen; Invitrogen) and inserted into pBR322 by gap repair (15). The two chimeric primers used to generate a retrieval vector were (5′-TGCGAGACTGTCAATTGCGCGTTCTA- TGCGAGACTGTCAATTGCGCGTTCTA-3′). The 5′ ends of each primer contained 51 nucleotides of sequence (capital letters) homologous to each of the two ends of the L2dtl region to be subcloned. At the 3′ ends were 19–21 nucleotides (italicized letters) homologous to pBR322. The PCR-derived vector with a pBR322 backbone was transformed into EL350 (15). Retrieval of the 10-kb L2dtl genomic sequence was dependent on the occurrence of homologous recombination between the 51 nucleotides at the free ends of the linear pBR322 vector and the homologous L2dtl sequences carried on the BAC.

The 10-kb genomic fragment was used to generate a targeting vector based on pKO-loxP (16). The knock-out strategy was to delete half of the L2dtl exon 1 (Fig. 2A). The 2.0-kb XbaI-SeqI fragment (short arm) and 4.8-kb Smal-HindIII fragment (long arm) were rendered blunt, ligated to appropriate linkers, and inserted into the pKO-loxP targeting vector at the unique XhoI and KpnI sites. The resultant targeting construct was then linearized by Ascl digestion and used for transfection of the RI ES cell line (a generous gift from A. Nagy, ES Core Facility, Ontario, Canada) (17) by electroporation as previously described (18). G418 (2 μM) and gancyclovir (10 μM) were used for transformant selection. Surviving cell colonies were isolated, established as clones, and genotyped by Southern blotting to ensure that homologous recombination had taken place (19).

Southern Blot Analysis—To genotype ES cell lines and identify targeted clones, 10 μg of genomic DNA was digested with PstI, separated in a 0.5% agarose gel in 1× Tris borate-EDTA buffer, blotted onto a nylon membrane (Millipore, Bedford, MA), and probed with a 360-bp PCR fragment that hybridized in an upstream portion of the targeted region of L2dtl. The 3′ probe, a 0.3-kb HindIII-NcoI fragment, hybridized to a downstream portion of the targeted region near exon 2.

Generation and Genotyping of L2dtl-deficient Mice—The correctly targeted ES cell clones were subsequently introduced into blastocysts of C57BL/6j mice by microinjection using a previously described technique (19). Chimeric mice were bred with wild-type C57BL/6j mice to obtain heterozygous (F1) mice, which were intercrossed to generate F2 mice. To genotype F1 and F2 weaned pups, genomic DNA was extracted from −0.5 cm of mouse tail and analyzed by PCR. The oligonucleotide primer pGKPDP20 (5′-ACTGCGTCTGGGAAAGGCGCCT-3′) hybridized only to the L2dtl knock-out allele, primer DE1F (5′-TGAGGAACGTGTGAAAGGCC-3′) hybridized only to the wild-type allele, and primer E1R (5′-CGCGCCGGCTGGAAGAGA-3′) hybridized both to the wild-type and L2dtl knock-out alleles (Fig. 1D). Primers pGKP and E1R amplified a 400-bp fragment from the L2DTL knock-out allele, and primers DE1F and E1R produced a 400-bp fragment from the wild-type allele. Briefly, in a 50-μl reaction volume, 500 ng of genomic DNA and 20 pmol of each primer (pGKP, DE1F, and E1R) were amplified in PCR buffer supplemented with 1.5 mM MgCl2, a 0.2 mM concentration of each dNTP, and 0.5 units of Taq polymerase (Bertaq). The cycling parameters were 95 °C for 5 min; 35 cycles of 1 min at 94 °C, 30 s at 55 °C, and 60 s at 72 °C; and a final extension for 7 min at 72 °C. Ten microliters of each reaction mixture was separated on a 1.0% agarose gel in 1× Tris acetate-EDTA buffer.

Embryo genotyping at 8.5, 10.5, and 12.5 days postcoitum (dpc) was performed by PCR analysis of DNA taken from either the yolk sac or the whole embryo. Tissues were digested overnight in 600 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) at 55 °C. DNA was purified in a protein precipitation solution followed by isopropyl alcohol precipitation and dissolved in 50 μl.
of 5 mM Tris-HCl (pH 8.0). The purified DNA (0.5 μg) was used for PCR as described above.  

**Timed Pregnancies**—To generate timed pregnancies, L2dtl<sup>−/−</sup> females received an intraperitoneal injection of serum gonadotropin from a pregnant mare (5 IU/animal; Sigma), followed 48 h later by human chorionic gonadotropin (5 IU/animal; Sigma). These mice were then allowed to mate with L2dtl<sup>−/−</sup> males. The females were removed the next morning, and the females were examined for the presence of vaginal plugs. Plugged females were sacrificed at 1.5, 2.5, 3.5, 8.5, 10.5, and 12.5 dpc to isolate embryos, which were then genotyped by PCR as described above (for E8.5, E10.5, and E12.5) and below (for E1.5, E2.5, and E3.5).  

**Genotyping of Preimplantation Stage Embryos**—Nested PCR was used to genotype E0.5, E1.5, E2.5, and E3.5 embryos. Individual embryos (or a single cell) (20) were immersed in 5 μl of alkaline lysis buffer to lyse the embryo (or cell), after which 5 μl of neutralization buffer was added before storage at −70 °C until the following PCR. The first round of amplification was carried out using the “HotStarTag Master Mix” kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For the first round, primers were mixed with PGKPD460 (5′-CAGAGGCCACCTTGTGTAGCGC-3′), E1F (5′-TCTTA-GTGGCGGGAGTTGGAG-3′), and E1R. An initial step at 95 °C for 15 min was followed by 35 cycles of 95 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. For the second round of amplification, 5 μl of the first round reaction mixture was added to two separate reaction mixes, each containing two different internal primers for the wild-type and knock-out alleles. The wild-type primers were second E1F (5′-TTTGAGGAGGTGTCAGACGCG-3′) and second E1R (5′-ATGCGCGCTGGTGCACAACGA-3′), whereas the knock-out allele primers were PGKPD520 and second E1R. Second round reactions were performed in a 50-μl reaction volume with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 100 μM each dNTP, 150 μM each primer, and 1.25 units of Taq polymerase (Invitrogen). The cycling program consisted of 35 cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, followed by a 10-min incubation at 72 °C.  

**Construction of the siRNA Expression Vector**—The mouse U6 promoter used in the siRNA expression vector was obtained from PCR with mouse genomic DNA. The siRNA-L2dtl target sequence, 5′-AAATCTGGCGCTTGAATAGAG-3′, was subcloned into the RNai-Ready pSIREN-RetroO vector (BD Biosciences Clontech), and the construct was designated siRNA-L2dtl. The DNA sequence of the construct was confirmed by sequencing using an automated DNA sequencer (PerkinElmer Life Sciences). The pSIREN-RetroQ-Luc vector (luciferase siRNA target; BD Biosciences Clontech) was used as a negative control, and injection buffer was used as the mock injection control. The siRNA vectors were prepared according to the manufacturer’s protocol (Qiagen), purified by centrifugation at 13,200 × g for 10 min, and stored in the injection buffer (5 mM Tris-HCl (pH 7.4), 0.1 mM EDTA) at −20 °C until used.  

**Embryo Collection, Microinjection, and Culturing for siRNA Analysis**—Mouse embryos were obtained from FVB superovulated female mice mated with FVB males. The female mice (~3–4 weeks old) were superovulated and mated as described above. Fertilized two-cell stage embryos were collected from the mated females 36–38 h after injection of hCG and cultured in KSOM (Chemicon, Temecula, Canada) under 5% CO<sub>2</sub> at 37 °C. Culture conditions and media have been described in detail elsewhere (21, 22). The siRNA vector was diluted in injection buffer to a final concentration of 4 and 8 ng/μl. An appropriate amount of siRNA vector was microinjected into the two nuclei (23) or one nucleus of the two-cell embryos. Embryos microinjected with the same volume of siRNA vector only or injection buffer only were used as sham controls. All microinjections were performed under a Leica inverted microscope with a micromanipulator (Leica Microsystems, Bensheim, Germany) using an automated Eppendorf microinjector (Eppendorf AG, Hamburg, Germany) and defined injection needles (Eppendorf AG). In subsequent experiments, a final concentration of 4 ng/μl of the siRNA vector was used unless otherwise stated. All injected embryos were allowed to develop to the blastocyst stage in culture. The growth patterns of embryos were microscopically examined and photographed.  

**Embryonic Semiquantitative RT-PCR**—Semiquantitative RT-PCR was performed using the OneStep RT-PCR kit (Qiagen). Three or five embryos were collected in 10 μl of RNase-free H<sub>2</sub>O and 10 μl of 5× PCR buffer and stored at −80 °C. The one-step RT-PCR was carried out in the same reaction tube in 1× Qiagen OneStep RT-PCR buffer, a 400 μM concentration of each dNTP, 1× Qiagen Q-Solution, 0.6 μM each primer, and 5 units of HotStart Taq polymerase mix. The initial RT step was 50 °C for 30 min, followed by 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min; and 72 °C for 10 min. For the second round of amplification, 5 μl of the first round reaction mixture was added to two separate reaction mixes, each containing two different L2dtl and Gapdh internal primers. Second round reactions were performed in a 50-μl reaction volume with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 100 μM each dNTP, 150 μM each primer, and 1.25 units of Taq polymerase (Invitrogen). The cycling program consisted of 26 cycles of 95 °C for 2 min, 55 °C for 30 s, and 72 °C for 1 min followed by a 10-min incubation at 72 °C.  

**Western Blot Analysis**—Embryos treated with siRNA and cultured to the blastocyst stage were washed five times in bovine serum albumin-free M2 medium (Sigma). Fifteen embryos were collected in 25 μl of 1× SDS sample buffer. After heat denaturation, the protein samples were stored at −80 °C until used. The proteins were resolved by SDS-polyacrylamide gels, transferred to a polyvinylidene fluoride membrane (Millipore), blocked with 5% milk in TBS (150 mM NaCl, 10 mM Tris-HCl (pH 7.5)) containing 0.1% Tween 20, and probed with anti-L2DTL antibody<sup>4</sup> and anti-Hsp90 antibody (Lab Vision, Fremont, CA). Following incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Amer sham Biosciences), immunoblots were visualized on RX-U x-ray film (Eastman Kodak Co.).  

**Nuclear Morphology of Embryos**—After siRNA experiments, embryos were fixed in phosphate-buffered saline with 4% paraformaldehyde for 20 min at room temperature followed by three washes for 5 min each in phosphate-buffered saline with 0.1% Tween 20 (PBST). Blocking and permeabilization were
carried out in 10 mg/ml bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline for 30 min at room temperature. Nuclei were stained with DAPI (1:2000) for 1 min, followed by three 5-min washes in PBST. Embryos were mounted on slides after DAPI nuclear counterstaining.

Transfections, Synchronization, and Multinucleated Cell Detection—RNAi oligonucleotides were synthesized by MoleculA Co. (Columbia, MD). The RNAi oligonucleotides corresponded to nucleotides 651–669 of the human L2DTL coding region (GenBank™ accession number AF195769). The negative control RNAi was purchased from Ambion (Austin, TX). In vitro transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. After RNAi oligonucleotide transfection, HeLa cells were synchronized at the beginning of the S phase by a double thymidine block and release. Ten hours after release (a total of 72 h after frame corresponding to a protein of 729 amino acids. The deduced amino acid sequence is shown in Fig. 1B. The ATG start codon was located at position 105, the TAG codon was at position 2294, and a putative nuclear localization signal was found at amino acid positions 197–203. The sequence also included a proline-rich motif (residues 636–640) that is highly homologous to the PXXP Src homology 3-binding consensus, and a putative LXXLL motif (residues 25–29). In addition, there was a leucine zipper at 575 and five WD repeats at positions 89, 131, 207, 306, and 350. Comparisons between Mus musculus and Homo sapiens indicate that the predicted homologous functional domains are notably conserved between the two species. Importantly, the Drosophila l(2)dtl cDNA, which is 2274 bp in length and encodes 758 amino acids, also shares the five WD40 repeats and the D-box, KEN-box, nuclear localization signal, and PEST sequences (Fig. 1, A and B).

RESULTS

Genomic Structure and Amino Acid Sequence Analysis of L2dtl—After the comparison of human L2DTL and the Celera mouse genome data base of strain 129/sv, 5′ and 3′ end-specific primers were designed for the putative L2dtl sequence. The 2.4-kb full-length open reading frame of L2dtl was cloned from the cDNA of murine fetal liver. The sequences of the exon and intron gaps were completed with the sequence of the L2dtl gene identified from the Celera data base. The deduced mouse gene contains 15 exons and spans 60 kb of genomic sequence on BAC A-589H1 (Fig. 1A). The cDNA contained a single open reading
L2dtl Is Expressed in Mouse Embryos and Highly Proliferative Postnatal Organs—To elucidate potential function(s) of L2dtl, we examined the expression profile of L2dtl in both embryos and postnatal mouse tissues by semiquantitative RT-PCR. During embryogenesis, L2dtl mRNA was highly expressed in the oocyte and in the E1–E12.5 embryos, but the levels were very low after E14.5 (Fig. 2A).

L2dtl mRNA expression was then examined in fetal and adult mouse tissues. Among the tissues examined, including brain, cerebellum, thymus, lung, heart, liver, stomach, pancreas, spleen, kidney, ovary, and testis, L2dtl was only expressed in low levels in the lymphoid organs (i.e. thymus and spleen). The adult testis was unique in the presence of a high level of L2dtl mRNA expression (Fig. 2B). All three organs are highly proliferative in adult mice, particularly the testis.

Generation and Characterization of L2dtl Knock-out Mice—Mouse ES cell lines with a truncated mutant L2dtl allele were generated by homologous recombination and used to study the in vivo function of L2dtl (Fig. 3B). The prediction was that this targeted disruption would result in the deletion of amino acids 4–17 and a frameshift, leading to early termination of L2dtl protein translation. Southern blot analysis with a probe corresponding to the upstream targeted region of L2dtl (5′ probe; Fig. 3, A and B) identified a 2.4-kb wild-type fragment and a 2.8-kb fragment for the targeted allele after PstI digestion. Another probe derived from the downstream portion of the targeted region (3′ probe; Fig. 3, A and B) yielded BstXI fragments for the wild-type (9.6-kb) and targeted (9.0-kb) alleles. Five of 282 clones were confirmed to have undergone homologous recombination (Fig. 3B). Five chimeric male mice were generated and bred with C57BL/6J mice to obtain germ line transmission of the generated L2dtl knock-out (KO) allele.

Offspring hemizygous for the L2dtl-KO allele were intercrossed, and 351 weaned pups were genotyped by PCR analysis (Table 1, Fig. 3C). No pups homozygous for the L2dtl-KO allele could be identified, indicating that pups lacking a functional L2dtl are not viable. The ratio of wild-type to hemizygous pups was about 1:2, as would be predicted from Mendelian inheritance. Hemizygous animals were grossly normal, and there was no overt phenotypic difference between hemizygotes and wild-type animals (data not shown). To determine at which stage of development the homozgyous mutant embryos died, 8.5-, 10.5-, and 12.5-dpc embryos from heterozygote intercrosses were isolated by timed pregnancies, and the collected two-cell, four-cell, 2.5-dpc, and 3.5-dpc embryos were genotyped by competitive PCR assay, WT and KO, PCR products amplified from the wild-type and targeted L2DTL allele, respectively.

In Vitro Analysis of Early Embryos—The failure of L2dtl-KO embryos to develop past 8.5 dpc suggests that L2dtl is required for very early embryogenesis. To assess the role of L2dtl in early embryogenesis, 0.5-, 1.5-, 2.5-, and 3.5-dpc embryos from heterozygote intercrosses were isolated by timed pregnancies, and the collected two-cell, four-cell, 2.5-dpc, and 3.5-dpc embryos were genotyped by single-cell PCR (Fig. 4, A–C). Homozygotes were found only among the 1.5-dpc embryos, an indication that homozygous L2dtl mutants die around or shortly after 1.5-dpc.
Phenotypic Analysis of L2dtl-deficient Embryos by Nuclear Microinjection of an siRNA Vector—The phenotypes of L2dtl-deficient embryos were examined after induction by RNAi via microinjection of the siRNA-L2dtl vector into eggs at the two-cell stage (23). This vector was able to suppress L2DTL mRNA expression in human HCC cell lines by more than 50% (data not shown). Each vector was microinjected into the nuclei of two-cell embryos, which were then allowed to develop in culture to the blastocyst stage. Embryos injected with siRNA-L2dtl showed a marked decrease in L2dtl expression at both the mRNA and protein levels (Fig. 5, A and B).

The phenotypes of the injected embryos were documented 24, 48, and 72 h after the microinjection (data not shown). After 72 h in culture, 77.1% of buffer-injected and 72.3% of siRNA vector only-injected embryos formed normal blastocysts. In contrast, siRNA-L2dtl-injected embryos frequently produced deformed blastocysts, and only 41.7% of the embryos were able to normally progress to the blastocyst stage (Table 2, Fig. 5C). To determine whether the siRNA knockdown effect is related to cell autonomy, one nucleus of the two-cell embryos was injected. After 72 h in culture, 75.1% of buffer-injected, 77.6% of siRNA vector only-injected, and 70.0% of siRNA-L2dtl-injected embryos formed normal blastocysts, similar to the control groups receiving injection into two nuclei (Table 2, Fig. 5C).

The nuclear morphology of the embryos at different time points after nuclear microinjection of the siRNA-L2dtl vector was examined to elucidate the mechanism of early embryonic lethality in L2dtl knock-out mice. No significant phenotypic changes in cell or nuclear morphology were observed among the three groups of embryos at 48 h. However, embryos that received siRNA-L2dtl exhibited evident cell lysis and karyorrhexis 72 h after injection. The injected embryos were further evaluated at an additional two time points, 54 and 62 h. Although normal mitotic figures were often observed in control embryos receiving vector or injection buffer, fragmentation of the mitotic chromosomes and chromosome lagging, indicative of mitotic catastrophe, were observed in the siRNA-L2dtl embryos (Fig. 6, A and B). The number of nuclei also dramatically decreased in the embryos administered siRNA-L2dtl, and many of the remaining nuclei were deformed (Fig. 6, A and B).

The mean number of mitotic figures in the L2dtl siRNA group significantly decreased compared with that observed in the control groups, \( p = 0.0001 \) (Fig. 6C).

Reduction of Human L2DTL Expression by RNAi Oligonucleotides Leads to an Increase in the Population of Multinucleated Cells in HeLa Cells—To further elucidate the physiological role of the L2DTL gene in human cells, particularly in cell cycle progression, L2DTL gene knockdown with RNAi oligonucleotides was applied in HeLa cells. After the treatment, the cells underwent a double thymidine block, followed by release into normal medium. Cell morphology was examined 10 h later. In

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**FIGURE 4. Loss of L2dtl causes early termination of development in L2dtl"+/" embryos.** A, the genotypes of embryos at different developmental stages were determined by single cell PCR (530 bp for wild-type allele, 370 bp for targeted allele). B, analysis of early embryos from intercrosses of L2dtl"+/" mice. Embryos were collected, photographed, and genotyped by single-cell PCR. C, microscopic analysis of E1.5 (two- or four-cell stage) to E3.5 embryos. Representative features are depicted. No L2dtl"-/" homozygote embryos were identified above the eight-cell stage.
the HeLa cells with L2DTL RNAi transfection, multinucleated cells significantly increased by 4-fold, \( p = 0.007 \) (Fig. 7, A and B). Western blotting showed that a reduction of L2DTL expression led to a dramatic decrease in the levels of PTTG1, phosphatidylinositol 3-kinase-p85, and proliferating cell nuclear antigen proteins (Fig. 7C).

**DISCUSSION**

The mouse L2dtl gene encodes a protein of 729 amino acids. This gene is 89% homologous with human L2DTL and 25% homologous with Drosophila l(2)dtl. Inter-species comparisons of the predicted proteins of L2DTL, L2dtl, and l(2)dtl indicate a cross-species conservation among these proteins. The mouse L2dtl shares all of its important motifs with the human L2DTL protein (Fig. 1A). Sequence comparison indicates that human L2DTL and mouse L2dtl are orthologs of the Drosophila l(2)dtl and produce WD40 repeat proteins. The WD motif (also known as the Trp-Asp or WD40 motif) is found in a multitude of eukaryotic proteins involved in a variety of cellular processes, such as signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, RNA processing, chromatin modification, and transcriptional mechanisms (24, 25). Given the diverse functions of WD repeat proteins, members of this family can play important roles in cellular differentiation and embryonic tissue development and maturation.

The WD repeat proteins C21 and Srw1 are involved in cell differentiation (26–28), and AAC3, which contains five WD repeats, is developmentally regulated (29). Another WD repeat protein, PWP1, is involved in growth; PWP1 null mutants grow slowly (30). Further, L2dtl has a long 3’-untranslated region of 1878 bp. Many genes with long 3’-untranslated regions play a regulatory role during development (27). Indeed, human L2DTL protein is expressed in multiple fetal tissues, with very low levels in the adult tissues, a phenomenon similar to its DRDOPHila counterpart (1). However, L2dtl expression during embryogenesis has not been investigated. In this study, we demonstrated for the first time that L2dtl mRNA is abundantly expressed in oocytes, E1–E12.5 embryos, and the testis as well as in some lymphoid organs (Fig. 2). In contrast, L2dtl mRNA levels were very low in most organs of newborn and adult mice. These findings suggest that L2dtl plays an important role in cell proliferation during embryogenesis, including in the germ cells, and its shut down is associated with tissue maturation and cell differentiation. Consistent with this suggestion, murine L2dtl

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**FIGURE 5.** L2dtl siRNA knockdown markedly decreases L2dtl expression and blocks embryonic development. After injection of the siRNA-L2dtl expression vectors into the nuclei of two-cell stage embryos, L2dtl expression and morphological phenotypes were examined at the blastocyst stage after 72 h in culture. A, semiquantitative RT-PCR analysis. L2dtl mRNA was analyzed for each injected embryos by single cell RT-PCR. Final product size is 690 bp. GAPDH was used as an internal control.

**TABLE 2**

In vitro development effects of L2dtl siRNA knockdown via injection of one and two nuclei of two-cell stage mouse embryos

| Treatment                  | Number of blastocysts 72 h after injection |
|----------------------------|--------------------------------------------|
|                            | Expt. 1 | Expt. 2 | Expt. 3 | Mean ± S.E |
| Injection of two nuclei    |         |         |         |            |
| 4 ng/μl plasmid            | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| Injection buffer           | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| siRNA L2dtl + injection    | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| buffer                     | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| 8 ng/μl plasmid            | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| Injection buffer           | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| Vector + injection buffer  | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| siRNA L2dtl + injection    | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| buffer                     | 4.7     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |

| Injection of one nucleus   |         |         |         |            |
| 4 ng/μl plasmid            | 7.3     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| Injection buffer           | 7.3     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| Vector + injection buffer  | 7.3     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| siRNA L2dtl + injection    | 7.3     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |
| buffer                     | 7.3     | 7.5     | 7.3     | 7.1 ± 3.3<sup>a</sup> |

<sup>a</sup> \( p = 0.001 \)

<sup>b</sup> \( p = 0.006 \)

<sup>c</sup> \( p = 0.005 \)

<sup>d</sup> \( p = 0.006 \)
**Embryonic Lethality in L2dtl Knock-out Mice**

expression was up-regulated during the early stage of liver regeneration after a two-thirds partial hepatectomy (data not shown), and L2DTL expression significantly decreased upon retinoic acid-induced neuronal differentiation of teratocarcinoma NT2 cells (2).

To elucidate the *in vivo* function of *L2dtl* in embryogenesis, we generated mice with disrupted *L2dtl* genes. We failed to obtain viable *L2dtl* homozygotes among the 351 neonates examined, whereas viable offspring showed a 1:2 ratio for *L2dtl*+/+ wild-type homozygotes and *L2dtl*+/− heterozygotes. L2DTL protein expression was detected in all of the viable *L2dtl*+/+ and *L2dtl*+/− embryos by immunofluorescence using a specific anti-L2dtl antibody (data not shown). These data indicate that *L2dtl*−/− homozygotes die during embryogenesis. Embryos were then collected from timed pregnancies, but we did not find any viable *L2dtl*−/− embryos beyond the 1.5-dpc stage among the 138 embryos examined (Fig. 4B). The conclusion, therefore, is that *L2dtl*−/− embryos abort development at the 4–8-cell stage. These observations provide the first *in vivo* genetic evidence that *L2dtl* is essential for early embryogenesis in mice.

The failure of mouse embryos lacking the *L2dtl* gene to develop beyond the eight-cell stage (Fig. 4C) differs from the larval lethality of mutant *Drosophila* l(2)dtl (1) and requires an explanation. In metazoans, an accumulation of maternal factors in the ooplasm during oocyte growth is responsible for the control of maturation, fertilization, and initial development of the newly formed embryo (31, 32). For example, maternal stocks of topoisomerase I and II are only present until the two- or four-cell stage, such that topoisomerase I−/− and II−/− embryos grow to 2–4-cell stage but then die (33). In *Drosophila*, embryogenesis is initiated by 13 rapid syncytial mitotic divisions that depend upon maternal factors and does not require zygotic gene activity. This maternally directed cleavage phase of development terminates at the midblastula transition, at which point the cell cycle slows down dramatically (34). Based on the presence of *L2dtl* mRNA and protein in oocytes and fertilized eggs, we postulated that mouse embryos lacking *L2dtl* could develop up to the 4–8-cell stage dependent upon the maternal stocks of the protein. Consistent with this hypothesis, we showed that *L2dtl* knockdown blastocysts showed frequent chromosome lagging, dispersion, fragmentation, and condensation of mitotic chromosomes (arrows in the upper panel), suggestive of mitotic catastrophe. Nuclei were labeled with DAPI 48, 54, 62, and 72 h after injection of the siRNA-L2dtl expression vector at two doses (4 and 8 ng/μl) into the two nuclei markedly inhibited *L2dtl* mRNA expression and resulted in a significant reduction in

**FIGURE 6. The phenotype and nuclear morphology of blastocysts 48–72 h after the nuclear injection of different vectors into two-cell stage embryos.** A, upper panel, smaller and fewer nuclei were observed in the embryos that received the siRNA-L2dtl expression vector when compared with those with vector-only (middle panel) or injection buffer only (mock-injected, lower panel). B, mitotic figures (arrows in lower panel) were often observed in the two control groups, whereas the L2dtl knockdown blastocysts showed frequent chromosome lagging, dispersion, fragmentation, and condensation of mitotic chromosomes (arrows in the upper panel), suggestive of mitotic catastrophe. Nuclei were labeled with DAPI 48, 54, 62, and 72 h after *in vitro* culture. C, mitotic figures were counted and statistically analyzed in each group. In the L2dtl siRNA group, there was a significant decrease in the number of mitotic figures, *p* = 0.0001.
fully developed blastocysts ($p = 0.001$) (Fig. 5C). For further clarification, the siRNA-L2dtl expression vector was microinjected into the one nucleus of the two-cell (single) embryos, with the result that embryonic development was unaffected (Fig. 5C, Table 2). Hence, our observations suggest that L2dtl functions in an exclusively cell-autonomous manner to regulate embryonic development (36). These results support the hypothesis that L2DTL is essential for early embryogenesis, but the mechanisms of the knock-out lethality remain unclear.

Examinations of the nuclear morphology of siRNA-L2dtl knockdown embryos exhibited fragmentation, condensation, chromosome lagging, and dispersal, indicative of mitotic catastrophe, which eventually led to termination of embryonic growth and lysis of the embryo cells (Fig. 6). Further, down-regulation of human L2DTL by RNAi oligonucleotides in HeLa cells led to the formation of multiple nuclei, a feature suggestive of cytokinesis failure. Hence, the molecular mechanisms for the disrupted cell cycle progression in L2dtl knock-out mice and L2DTL knockdown cells deserve further consideration.

Amino acid sequence analysis revealed that human L2DTL and mouse L2dtl, and even Drosophila l(2)dtl, possess several important motifs crucial for cell cycle progression, including a D-box and KEN-box (37–40). Our concurrent in vitro studies confirmed that human L2DTL was a cell cycle-regulated nuclear protein and, importantly, also a novel member of the centrosome proteins, with co-localization and co-fractionation with γ-tubulin in double immunofluorescence and centrosome isolation assays. Furthermore, down-regulation of human L2DTL by RNAi oligonucleotides in HeLa cells was associated with decreased expression levels of phosphatidylinositol 3-kinase-p85, proliferating cell nuclear antigen, and PTTG1/securin proteins (Fig. 7). PTTG1 acts as an anaphase inhibitory protein to prevent abnormal chromosome segregation (41). We therefore speculated that L2dtl knockdown would interfere with cell division and cytokinesis. Together with the RNAi knockdown of the L2dtl function in the mouse embryos showing signs of mitotic catastrophe of the chromosomes and termination of embryonic growth and lysis of embryo cells (Fig. 6), these findings led us to suggest that a knock-out of the pivotal L2dtl protein leads to impaired DNA synthesis and cell cycle progression and abnormal chromosome segregation, probably through the genetic alterations of many crucial cell cycle-regulated genes, eventually ending in embryonic lethality.

In conclusion, our findings demonstrated that the L2dtl gene is essential for early mouse embryogenesis, and targeted disruption of L2dtl gene expression leads to embryonic lethality in the preimplantation stage. Developmental arrest can be recreated with siRNA knockdown of L2dtl by microinjection into the nuclei of two-cell stage embryos, in which the lack of L2dtl leads to mitotic catastrophe, failure of cell cycle progression, and, hence, embryonic lethality.

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Embryonic Lethality in L2dtl Knock-out Mice

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