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

Chao-Lien Liu¹, I-Shing Yu², Hung-Wei Pan¹, Shu-Wha Lin²,³, and Hey-Chi Hsu¹,⁴

¹Graduate Institute of Pathology and ²Department of Clinical Laboratory Sciences and Medical Biotechnology; College of Medicine, National Taiwan University; ³Departments of Laboratory Medicine and ⁴Pathology, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan.

Address reprint requests to: Prof. Hey-Chi Hsu, Department of Pathology, National Taiwan University Hospital. e-mail: heychi@ha.mc.ntu.edu.tw; fax: (886-2) 23410876; and Prof. Shu-Wha Lin, Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei, Taiwan. e-mail: swlin@ha.mc.ntu.edu.tw; fax: (886-2) 23817083.

Supported by grants from the National Science Council of the Republic of China (NSC93-2320-B002-108 and NHRI-EX94-9427NI to HCH; NSC94-3112-B002-014, NSC95-3112-B002-017 and 91-B-FA09-2-4 to SWL), Taipei, Taiwan.

Running title: Embryonic lethal in L2dtl knockout mice

**ABSTRACT**

Lethal (2) denticleless (l(2)dtl), 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 the *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 knockout resulted in early embryonic lethality. *L2dtl*⁻/⁻ embryos were deformed and terminated development at the 4- to 8-cell stage. Microinjection of an siRNA vector (siRNA-L2dtl) into the 2-cell stage nuclei of wild-type mouse embryos led to the cell cycle progression failure, termination of cell division and, eventually, embryonic death during the pre-implantation 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.

http://www.jbc.org/cgi/doi/10.1074/jbc.M606535200 The latest version is at JBC Papers in Press. Published on November 15, 2006 as Manuscript M606535200

Copyright 2006 by The American Society for Biochemistry and Molecular Biology, Inc.
However, the embryos developed normally after microinjected into one nucleus of the two-celled embryos. The siRNA studies in HeLa cells showed that L2dtl protein depletion results in multi-nucleation and down-regulation of PI3K, PCNA 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.

**Key words:** L2dtl, development, lethal, siRNA knockdown, cell division.

**INTRODUCTION**

The lethal (2) denteicleless (l(2)dtl) gene in *Drosophila melanogaster* encodes a unique 83 kDa protein that is upregulated 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 no. 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 WD-40 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 (Pan et al., submitted). Despite these observations, the physiological function of L2DTL, particularly its role in embryogenesis, has not been resolved. Our bioinformatic analysis (unpublished) showed that the amino acid sequence of human L2DTL is 89% homologous to mouse L2dtl (GenBank Accession no. NM-029766) and 25% homologous to *Drosophila* l(2)dtl (GenBank Accession no. 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 (NLS), a D-box, a KEN-box and PEST sequences, are conserved.
The goal of this study was to investigate the physiological roles of \textit{L2dtl}, an apparent ortholog of \textit{l(2)dtl}, in embryonic mouse development. Homologous recombination in mouse embryonic stem (ES) cells was used to generate \textit{L2dtl} knockout mice. Initial breeding and characterization revealed the embryonic lethality of \textit{L2dtl} null mice. The development of \textit{L2dtl} null embryos ceased at the 4-cell to 8-cell stage. A similar phenomenon was observed in 2-cell stage embryos using siRNA knockdown of the \textit{L2dtl} transcript. The results indicate that \textit{L2dtl} is required for early embryonic development in mice.

\textbf{MATERIALS AND METHODS}

\textit{Computer analysis of the mouse \textit{L2dtl} gene.}

The genomic sequence of \textit{L2dtl} was obtained from strain 129 in the Celera mouse database. The sequence revealed a possible \textit{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 \textit{L2dtl} 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 \textit{L2dtl} reference (Accession no. NM-029766). The 2.4 kb sequence was further analyzed using ELM (http://elm.eu.org/), SMART (http://smart.embl-heidelberg.de/), PSORT (http://www.psort.org/) and PESTfind (http://www.at.embnet.org/embnet/tools/bio/PESTfind/) software programs to deduce amino acid sequence and functional domains.

\textit{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 (Life Technologies, Carlsbad, CA, USA). After treatment with DNaseI, total RNA was transcribed with Superscript II (Gibco, Carlsbad, CA, USA) using oligo-dT primers. The primer pairs were: 5'-AACATTATGATCTGGGACACCAGG-3' and 5'-ATCACCTGGTTTCTCCTCTAGG-3' for \textit{L2dtl}, and 5'-GACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGGTTGCTGTAG-3' for \textit{GAPDH}. For each gene, the number of PCR cycles was optimized to detect differences in mRNA amounts: 30 cycles for \textit{GAPDH} and 35 cycles for \textit{L2dtl}. Each cycle consisted of 30 s at 95\(^\circ\)C, 40 s at 55\(^\circ\)C and 1 min at 72\(^\circ\)C using Taq polymerase (BerTag, DCB, Taipei, Taiwan). PCR products were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide.

\textit{Disruption of \textit{L2DTL} in mouse embryonic stem cells.}

A 10-kb \textit{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, California, USA) and inserted into pBR322 by gap-repair (15). The two chimeric primers used to generate a retrieval vector
were (5'-TAGTGGCTTTTATCCTGGCTTCTAG
TGTTACAGTTAGCTGAGTTGGAAACT
GCGGCCGCgatacgcgagcgaacgtga-3' and
5'-TGCGAGACTGTCAATTGCGGTCT
AAAGCAGGTTCCATGTGGATGAGAAC
AGTCGACttcttagcagttgac-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 knockout strategy was
to delete half of the L2dtl exon 1 (Fig. 2A).
The 2.0-kb XbaI-SepI fragment (short arm)
and 4.8-kb SmaI-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 byAscI
digestion and used for transfection of the R1
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
did not have 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,
USA), 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 (WT) 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 pGKPD520
(5'–ACTGCCTTGGGAAAAGCGCCT-3')
hybridized only to the
L2dtl knockout allele,
primer DE1F
(5'–TGAGGAACGGTGAGTAACGGC-3')
hybridized only to the wild-type allele, and
primer E1R
(5'–TGGCGCCTTAAGATTGAGTTC-3')
hybridized to both the wild-type and L2dtl
knockout alleles (Fig. 1D). Primers pGKPD
and E1R amplified a 400-bp fragment from the L2DTL-knockout 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 (pGKPD, DE1F and E1R) were amplified in PCR buffer supplemented with 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 0.5 U 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-dpc (days post coitum) 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 isopropanol 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+/– females received an intraperitoneal injection of serum gonadotropin from a pregnant mare (5 IU per animal; Sigma, St. Louis, MO, USA), followed 48 h later by human chorionic gonadotropin (5 IU per animal; Sigma). These mice were then allowed to mate with L2dtl+/– males. The males 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 pre-implantation 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 reaction. The first round of amplification was carried out using the "HotStarTag Master Mix" Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. For the first round, primers were mixed with PGKPD460 (2)

(5’–CAGAGGCCACTTGTGTAGCGC-3’), E1F
(5’–TCTTAGTGGCGGGAGTTGGAG-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 knockout alleles. The wild-type primers were second E1F
(5’–TTGTGGAGGGTGAGGCAGGCG-3’)
and second E1R
while the knockout 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 MgCl₂, 100 µM of each dNTP, 150 nM of each primer and 1.25 U Taq polymerase (Gibco Life Technologies, Valencia, CA, USA). 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 by PCR from mouse genomic DNA. The siRNA-L2dtl target sequence, 5’–AAATCTGGCGCTTGAATAGAG-3’, was subcloned into the RNAi-Ready pSIREN-RetroO vector (BD Biosciences Clontech, Bedford, MA, USA), and the construct was designated siRNA-L2dtl. The DNA sequence of the construct was confirmed by sequencing using an automated DNA sequencer (Perkin Elmer, Norwalk, CT, USA). The pSIREN-RetroQ-Luc vector (luciferase siRNA target, BD Biosciences Clontech, Bedford, MA, USA) was used as a negative control, and injection buffer as the mock-injection control. The siRNA vectors were prepared according to the manufacturer’s (Qiagen) protocol, purified by centrifugation at 13200×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 2-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% CO₂ 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 2-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 semi-quantitative RT-PCR.** Semi-quantitative RT-PCR was performed using the One-step RT-PCR Kit (Qiagen). Three or five embryos were collected in 10
µl of RNase-free H2O 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, 400 µM of each dNTP, 1× Qiagen Q-Solution, 0.6 µM of each primer and 5 U 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 mixtures 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 MgCl2, 100 µM of each dNTP, 150 nM of each primer and 1.25 U Taq polymerase (Gibco Life Technologies). 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 BSA-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 onto polyvinylidene fluoride a 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 (Pan et al., submitted) and anti-Hsp90 antibody (Lab Vision, Fremont, CA, USA). Following incubation with HRP-conjugated goat anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK), immunoblots were visualized on RX-U X-ray film (Kodak, New York, USA).

**Nuclear morphology of embryos.** After siRNA experiments, embryos were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 20 min at room temperature followed by three washes for 5 min each in PBS with 0.1% Tween-20 (PBST). Blocking and permeabilization were carried out in 10 mg/ml BSA and 0.1% Triton X-100 in PBS 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 multi-nucleated cell detection.** RNAi oligos were synthesized by MoleculA Company (Columbia, MD, USA). The RNAi oligos corresponded to nts 651-669 of the human L2DTL coding region (GeneBank accession no. AF195765). The negative control RNAi was purchased from Ambion (Austin, TX, USA). In vitro transfection was performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocols. After RNAi oligo 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 RNAi oligo transfection), the cells were stained with DAPI and the multi-nucleated cells counted.
under a microscope.

**Antibodies.** Commercially obtained antibodies included anti-α-tubulin (Sigma), anti-PCNA (FL-261) and PI3K-p85 (sc-1637) (Santa Cruz Biotechnology, Inc., CA, USA); Securin antibody (DCS-280, ab3305, Abcam, Inc., Cambridge, UK); and anti-rabbit and mouse IgG HRP-conjugated secondary antibodies (Roche Molecular Biochemicals, Mannheim, Germany).

**Statistical analysis.** Data were analyzed by ANOVA and Duncan’s multiple range tests. A value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Genomic structure, deduced amino acid sequence analysis of L2dtl.** After the comparison of human L2DTL and the Celera mouse genome database 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 database. 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 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 at position 2294, and a putative NLS 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 SH3-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, NLS and PEST sequences (Fig. 1A, B).

**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 semi-quantitative RT-PCR. During embryogenesis, L2dtl mRNA was highly expressed in the oocyte and in the embryonic day (E) 1–12.5 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**
**knockout 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 the 4th to 17th amino acids 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. 3A, 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. 3A, B) yielded BstXI fragments for the wild-type (9.6 kb) and targeted (9.0 kb) alleles. Five out 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 knockout (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 homozygous mutant embryos died, 8.5-, 10.5-, and 12.5-dpc embryos from heterozygote intercrosses were isolated and genotyped by PCR (Table 1). No homozygous mutant embryos were identified at these stages, indicating that L2dtl-KO embryos die prior to 8.5 dpc.

**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 2-cell, 4-cell, 2.5-dpc and 3.5-dpc embryos were genotyped by single-cell PCR (Fig. 4A-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 2-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 2-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. 5A, B).
The phenotypes of the injected embryos were documented 24, 48, and 72 h after the microinjection of siRNA. No phenotypic difference was observed among embryos receiving siRNA-L2dtl, empty vector and injection buffer cultured for 24 and 48 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 of 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 knockout mice. No significant phenotypic changes in cell or nuclear morphology were observed among the three groups of embryos at 48 hours. 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. While 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. 6A, B). The number of nuclei also dramatically decreased in the embryos administered siRNA-L2dtl, and many of the remaining nuclei were deformed (Fig. 6A, 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 oligos leads to increase in the population of multi-nucleated 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 oligos 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 the HeLa cells with L2DTL RNAi transfection, multi-nucleated cells significantly increased by 4 fold, \( P = 0.007 \) (Fig. 7A, 7B). Western blotting showed that a reduction of L2DTL expression led to a dramatic decrease in the levels of PTTG1, PI3K-p85, and PCNA 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%
with *Drosophila* l(2)dtl. Interspecies 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’ UTR of 1878 bp. Many genes with long 3’UTRs 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 *Drosophila* counterpart (1). But, 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, E1~12.5 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 expression was upregulated during the early stage of liver regeneration after two-thirds partial hepatectomy (data not shown), and L2DTL expression significantly decreased upon RA-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, while 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 8-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 are responsible for the control of maturation, fertilization and initial development of the newly formed embryo (31,32). For example, maternal stocks of topo I and II are only present until the 2- or 4-cell stage, such that topo 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 (MBT), 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 posited that mouse embryos lacking L2dtl could develop up to the 4- to 8-cell stage dependent upon the maternal stocks of the protein. Consistent with this hypothesis, we showed that L2dtl−/− embryos could indeed grow normally to the 4-8 cell stage. The morphology of the homozygous mutant embryos was examined at different time points for a clue to the cause of lethality. Phase contrast microscopy showed that L2dtl−/− embryos had morphologically normal nuclei before the 4- or 8-cell stage, whereas the nuclei of 3.5-dpc L2dtl−/− embryos appeared deformed or lysed, leading to abortion of development.

To further demonstrate the crucial role of L2dtl in the early division of embryonic cells, siRNA was used to knockdown L2dtl in fertilized oocytes at the 2-cell stage. It has been shown that the microinjection of a circular plasmid vector into the pronucleus allows the transient expression of a vector-encoded gene without integration into the genome (35). Microinjection of the siRNA-L2dtl expression vector at two doses (4 ng/µl and 8 ng/µl) into the two nuclei markedly inhibited L2dtl mRNA expression and resulted in a significant reduction in fully developed blastocysts (P = 0.001) (Fig. 5C). For further clarification, the siRNA-L2dtl expression vector was microinjected into one nucleus of the two-cell (single) embryos, with the result that embryonic development was unaffected (Fig. 5C; Table2). Hence, our observation 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 knockout lethality remain unclear.

Examinations of the nuclear morphology of siRNA-L2dtl vector injected embryos in more defined time periods showed that the 2-cell stage embryos receiving siRNA-L2dtl vector developed normally in culture for 48 h, but became deformed and subsequently failed to develop into blastocysts after 72 h
in culture. Over 54 and 62 h, frequent normal mitotic figures were observed in the control embryos receiving vector and buffer alone. In contrast, the mitotic chromosomes in the 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, downregulation of human L2DTL by RNAi oligos 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 knockout 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 (Pan et al., submitted). Furthermore, downregulation of human L2DTL by RNAi oligos in HeLa cells was associated with decreased expression levels of PI3K-p85, PCNA 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 knockout 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 pre-implantation stage. Developmental arrest can be recreated with siRNA knockdown of L2dtl by microinjection into the nuclei of 2-cell stage embryos, in which the lack of L2dtl leads to mitotic catastrophe, failure of cell cycle progression and, hence, embryonic lethality. National Science Council, National Taiwan University Hospital and the National Health Research Institute, Department of Health, the Republic of China, Taipei, Taiwan.

The authors wish to thank Dr. Shih-Yao Lin at AbGenomics Corporation for critical

ACKNOWLEDGEMENTS

These studies were supported by grants NSC93-2320-B002-108 and NHRI-EX94-9427NI to HCH; NSC95-3112-B002-017, 91-B-FA09-2-4 and 90A01~91A06 to SWL from the
reading of the manuscript and helpful discussions; Ms. Chiu-Wen Yeh, Yen-Lin Lin, and Jia-Yen Su for their excellent technical assistance; and the technical services provided by the TMMC facility of the National Research Program for Genomic Medicine, NSC.
REFERENCES

FIGURE LEGENDS

Fig. 1. Genomic structure of mouse L2dtl and phylogenetic comparison of the primary structure with Drosophila l(2)dtl and human L2DTL. A. Exons and protein primary structures are shown as filled and shaded boxes to highlight functional modules. Filled areas are 5’ and 3’ untranslated regions. The putative D-Box (macron), WD40 domain (gray boxes), nuclear-localization signal (filled bar), PEST sequence (virgule), and KEN-Box (straightedge) are shown. B. The deduced amino acid sequences of mouse L2dtl protein shown according to exons. The putative nuclear localization signal, WD repeats, LXXLL motif, leucine zipper pattern, D-Box, KEN-Box, PEST sequence and SH3-binding consensus are shown.

Fig. 2. Expression profile of L2dtl mRNA in mouse embryos and tissues.
Semi-quantitative RT-PCR analysis of wild-type mice: A. L2dtl mRNA expression in mouse oocytes, zygotes and embryos of different stages. B. L2dtl mRNA expression profiles in various tissues of adult mice. GAPDH served as an internal control.

Fig. 3. Generation of L2dtl knockout mice. A. Conventional KO construct designed to delete the first half of exon 1 of L2dtl genomic DNA by insertion of a Neo cassette. The pKO-loxP based targeting vector contains a 2.4 kb short isogenic arm 5’ of and a 4.8 kb long isogenic arm 3’ of the neo cassette. B. Genomic DNA from ES cells digested with PstI and probed with 360 bp 5’ probe to identify homologous recombinants. The positive clones (left) were verified by BstXI digestion and probed with a 3’ probe (right) to confirm homologous recombination events. C. Identification of L2dtl+/− and L2dtl +/− mice by competitive PCR assay. WT and KO indicate PCR products amplified from the wild-type (WT) and targeted (KO) L2DTL allele.

Fig. 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 (2- or 4-cell stage) to E3.5 embryos. Representative features are depicted. No L2dtl +/− homozygote embryos were identified above the 8-cell stage.

Fig. 5. L2dtl siRNA knockdown markedly decreases L2dtl expression and blocks embryonic development. After injection of the siRNA-L2dtl expression vectors into the nuclei of 2-cell stage embryos, L2dtl expression and morphological phenotypes were examined at the blastocyst stage after 72 h in culture. A. Semi-quantitative 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. B. Western blot analysis of L2dtl protein expression. L2dtl and its putative high molecular weight glycosylated form (arrows) dramatically decreased in embryos receiving the siRNA-L2dtl vector. Hsp90 was used as a loading control. Short exp and long exp: short and long exposures, respectively. C. Morphology of injected embryos cultured for 72 h. From left to right: embryos injected with siRNA-L2dtl vector (far left), showing deformed or lysed patterns, injected with vector alone (second) and injected with buffer alone (third), showing normal embryo pattern, and injected with the siRNA-L2dtl vector into one of the two nuclei of the two-cell embryos (single) (right), showing normal embryo pattern. All experiments were repeated at least three times (quantified and summarized in Table 2).

Fig. 6. The phenotype and nuclear morphology of blastocysts 48~72 hours 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 to 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 at 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, \( P = 0.0001 \).

Fig. 7. Downregulation of L2DTL by RNAi oligos increased multi-nucleation events in HeLa cells. A. HeLa cells after transfection with RNAi oligos were double blocked with 2 nM thymidine and released for 10 h before multi-nucleated cells (arrow) were counted. B. Graph shows the increased percentage of multi-nucleated cells after L2DTL RNAi oligo transfections (~4-fold greater than the control). C. Western blot of HeLa cell lysates, prepared 72 h after injection of L2DTL or control RNAi oligos, showed a downregulation of L2DTL, PI3K-p85, PTTG1, and PCNA proteins.
TABLE 1. Genotyping the progeny of heterozygous matings

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>At weaning</td>
<td>351</td>
<td>106</td>
<td>245</td>
<td>0</td>
</tr>
<tr>
<td>12.5-dpc embryos</td>
<td>11</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>10.5-dpc embryos</td>
<td>37</td>
<td>11</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>8.5-dpc embryos</td>
<td>27</td>
<td>11</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 2. *In vitro* development effects of *L2dtl* siRNA knockdown via injection of one and two nuclei of 2-cell stage mouse embryos

<p>| Treatment                      | No. of blastocysts 72 h after injection |</p>
<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Mean ± SEM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection of Two nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid (4 ng/µl):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection buffer</td>
<td>11/15</td>
<td>25/31</td>
<td>34/44</td>
<td>77.1±3.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vector + Injection buffer</td>
<td>11/16</td>
<td>25/31</td>
<td>27/40</td>
<td>72.3±7.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Si-RNA L2dtl + Injection buffer</td>
<td>7/17</td>
<td>18/37</td>
<td>12/34</td>
<td>41.7±6.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Plasmid (8 ng/µl):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection buffer</td>
<td>11/17</td>
<td>30/47</td>
<td>24/32</td>
<td>67.8±3.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vector + Injection buffer</td>
<td>10/16</td>
<td>20/30</td>
<td>14/24</td>
<td>62.5±2.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Si-RNA L2dtl + Injection buffer</td>
<td>7/18</td>
<td>20/42</td>
<td>13/34</td>
<td>41.6±3.0&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Injection of One nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid (4 ng/µl):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection buffer</td>
<td>29/37</td>
<td>29/37</td>
<td>22/32</td>
<td>75.1±5.5</td>
</tr>
<tr>
<td>Vector + Injection buffer</td>
<td>25/33</td>
<td>20/27</td>
<td>29/35</td>
<td>77.6±4.7</td>
</tr>
<tr>
<td>Si-RNA L2dtl + Injection buffer</td>
<td>24/31</td>
<td>20/32</td>
<td>28/40</td>
<td>70.0±7.5</td>
</tr>
</tbody>
</table>

Mean values with the same superscripts are significantly different as follows:

<sup>a</sup><sub>P = 0.001</sub>, <sup>b</sup><sub>P = 0.006</sub>, <sup>c</sup><sub>P = 0.005</sub>, <sup>d</sup><sub>P = 0.006</sub>. 

Downloaded From http://www.jbc.org/ by guest on July 18, 2017
Fig. 1
Fig. 2
Fig. 3
Fig. 4

(A) Gel showing WT and KO bands.

(B) Table showing genotype distribution across different stages.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>+/+</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5-dpc embryos</td>
<td>77</td>
<td>23</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>2.5-dpc embryos</td>
<td>61</td>
<td>11</td>
<td>41</td>
<td>9</td>
</tr>
<tr>
<td>1.5-dpc embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-cell stage</td>
<td>68</td>
<td>6</td>
<td>41</td>
<td>21</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>55</td>
<td>11</td>
<td>27</td>
<td>17</td>
</tr>
</tbody>
</table>

(C) Embryonic images at E3.5-dpc, E2.5-dpc, 4-cell, and 2-cell stages.
Fig. 5
Fig. 6
Fig. 7

A. DAPI

control

L2DTL-RNAi

phase-contrast

B. Multi-nucleated cells (%)

Control | L2DTL-RNAi

C. RNAi:

<table>
<thead>
<tr>
<th>Ctl</th>
<th>L2DTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2DTL</td>
<td></td>
</tr>
<tr>
<td>PTTG1</td>
<td></td>
</tr>
<tr>
<td>PI-3K p85</td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td></td>
</tr>
<tr>
<td>α-tub</td>
<td></td>
</tr>
</tbody>
</table>
L2dtl is essential for cell survival and nuclear division in early mouse embryonic development

Chao-Lien Liu, I-Shing Yu, Hung-Wei Pan, Shu-Wha Lin and Hey-Chi Hsu

J. Biol. Chem. published online November 15, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M606535200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2006/11/15/jbc.M606535200.citation.full.html#ref-list-1