Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation

Mahua Mukhopadhyay1,*, Andreas Teufel1,*, Tsuyoshi Yamashita1,†, Alan D. Agulnick1,‡, Lan Chen1, Karen M. Downs2, Alice Schindler1, Alexander Grinberg1, Sing-Ping Huang1, David Dorward3 and Heiner Westphal1,*

1Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA
2Department of Anatomy, University of Wisconsin-Madison Medical School, Madison, WI 53706, USA
3National Institutes of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840, USA
*These authors contributed equally to this work
†Present address: Department of Obstetrics and Gynecology, Asahikawa Medical College, Nishikagura 4-5-3-11, Asahikawa, Japan
‡Present address: CyThera Inc., 3550 General Atomics Court, San Diego, CA 92121, USA
§Author for correspondence (e-mail: hw@helix.nih.gov)

Accepted 18 October 2002

SUMMARY

The LIM domain-binding protein 1 (Ldb1) is found in multi-protein complexes containing various combinations of LIM-homeodomain, LIM-only, bHLH, GATA and Otx transcription factors. These proteins exert key functions during embryogenesis. Here we show that targeted deletion of the Ldb1 gene in mice results in a pleiotropic phenotype. There is no heart anlage and head structures are truncated anterior to the hindbrain. In about 40% of the mutants, posterior axis duplication is observed. There are also severe defects in mesoderm-derived extraembryonic structures, including the allantois, blood islands of the yolk sack, primordial germ cells and the amnion. Abnormal organizer gene expression during gastrulation may account for the observed axis defects in Ldb1 mutant embryos. The expression of several Wnt inhibitors is curtailed in the mutant, suggesting that Wnt pathways may be involved in axial patterning regulated by Ldb1.

Key words: Ldb1, Wnt inhibitor(s), Otx2, Mouse, Anterior-posterior axis

INTRODUCTION

Ldb1 (also called NL1 or Clim-2), a LIM domain-binding protein, was isolated based on its ability to bind LIM domains present in LIM-homeodomain (HD) and LIM-only proteins (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997). Ldb1 is thought to mediate transcriptional regulation by protein complexes consisting of LIM-HD and other classes of transcription factors such as GATA, bHLH and Otx (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997; Jurata and Gill, 1997; Visvader et al., 1997; Wadman et al., 1997; Breen et al., 1998; Dawid et al., 1998; Jurata and Gill, 1998). The protein-protein interactions with Ldb1 may not be restricted to proteins containing LIM domains since Chip, the Drosophila homolog of Ldb1, also interacts with homeodomain proteins lacking LIM domains. A separate domain of Chip that is distinct from the LIM-binding domain appears to be involved in this interaction with other homeodomain proteins (Torigoi et al., 2000).

Genes homologous to Ldb1 have been found in organisms as diverse as C. elegans and humans. While there are two distinct Ldb gene family members in vertebrates and four in zebrafish, only one member has been identified in C. elegans and D. melanogaster (Cassata et al., 2000). Gene duplication events are thought to be responsible for the increase in the number of both the transcription factors and interacting Ldb proteins during the course of evolution (Meyer and Schartl, 1999).

LIM domains, cysteine-rich motifs that bind zinc and mediate protein-protein interactions, are found in many proteins expressed in the embryo (reviewed by Bach, 2000; Dawid et al., 1998). The biological consequences of disruption of LIM-HD transcription factors are severe, demonstrating the importance of these genes in development (for review, see Hobert and Westphal, 2000). It is well established that LIM domains have a negative regulatory effect on the function of LIM-homeodomain proteins (German et al., 1992; Taira et al., 1994; Sanchez-Garcia and Rabbitts, 1994). Embryo microinjection studies in Xenopus have shown that interactions between LIM domains and Ldb1 help relieve the inhibitory effect of the LIM domains (Agulnick et al., 1996). The LIM-binding portion of Ldb1 has been localized to the carboxyl terminus, while the amino-terminal region is involved in homodimer formation (Breen et al., 1998). The later domain may also allow formation of heterodimers with Ldb2 (also called NL2 or Clim-1), the second member of the vertebrate Ldb family (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997).
The ubiquitous expression pattern of Ldb1 during development and its interaction with numerous transcriptional regulators (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997; Jurata and Gill, 1997; Visvader et al., 1997; Wadman et al., 1997; Breen et al., 1998; Dawid et al., 1998; Jurata and Gill, 1998) suggest a critical function of the gene in a variety of developmental pathways. In order to provide direct evidence for the role of mammalian Ldb1 in embryonic development, we have eliminated its function in mice. Ldb1 null mutants show severe anterior-posterior patterning defects that include anterior truncation, posterior duplication, and lack of heart and foregut formation. Functional impediments of the organizer genes Otx2, Lim1 (Lhx2), Dkk1, Hesx1 and Hnf3β during gastrulation may account for the anterior-posterior axis patterning defect observed in the mutant at post-gastrulation stages of development. Our results suggest that defects in the regulation of Wnt pathways may be the underlying cause for the observed mutant phenotype.

**MATERIALS AND METHODS**

**Generation of Ldb1+/– mutant mice**

Ldb1 genomic clones were isolated from a mouse 129/Sv genomic library (a gift from Dr. S. Tonegawa, Massachusetts Institute of Technology, Cambridge, MA). The targeting vector was constructed by replacing exon 3-9 of Ldb1 with a PGK-NEO cassette, preserving 4.3 kb (5′) and 2.4 kb (3′) of flanking homologous sequences and using the thymidine kinase gene for double selection. The final targeting vector was linearized at a unique NotI site before transfection of ES cells. Transfected ES cells were subjected to double selection. Targeted disruption of the Ldb1 gene via homologous recombination was confirmed by Southern blot analysis. Genomic DNA isolated from ES cells was digested with EcoRV or HindIII for Southern analysis. A 0.3 kb EcoRV-BglII fragment was used as a 5′ probe to identify a 6.2 kb fragment of the mutated allele. A 0.45 kb NcoI-NcoI fragment served as the 3′ probe to detect a 6.6 kb fragment of the mutated allele. Recombinant ES cells were injected into blastocysts to generate chimeric mice. Chimeric males were mated with C57BL/6 females to produce Ldb1+/– animals, which were intercrossed to produce Ldb1+/- offspring for analysis.

**Genotyping**

For genotypic analysis of E7.5 to E8.5 embryos, whole embryos were digested overnight upon completion of in situ analysis and photographed. The genotype of the embryos was determined by polymerase chain reaction using the following primers: Ldb1 wild-type allele primers 5′-CCATGGCCGACCTGATACCA-3′ and 5′-CTGGTTAACAATGGGTITGCGTGA-3′; Neo primers 5′-CTTGGGAGAGGCTATTC-3′ and 5′AGGTGAGATGACAGGAAGATC-3′, which yielded bands of 175 and 280 bp, respectively.

**Detection of maternal Ldb1 mRNA in oocytes**

Total RNA from unfertilized eggs was isolated using Picopure RNA isolation kit from Arcturus (Cat. no. KIT0202). RNA quantification was performed using RiboGreen RNA Quantification Reagent and a kit from Molecular Probes (Cat. nos. R-11491 and R-11490). About 15 ng of RNA was used for RT-PCR analysis. RT-PCR was performed using Smart Race cDNA Amplification kit from Clontech. Ldb1 primers used for RT-PCR analyses were as follows. Left primer 5′-GACAGGTCTGCTGTGTACAACT-3′, right primer 5′-CATAAGTTCTGATGCCTCTGACTA-3′. Nucleotide sequences of the control primer set: left primer 5′-GAACAGTCTTGACGTAAGTGCTCT-3′, right primer 5′-GTTACAGTCTTGACGTAAGTGCTCT-3′.

**Histology and in situ hybridization**

For histological analyses, embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned at 5 μm and stained with Hematoxylin and Eosin. In situ hybridization to whole embryos was performed as described previously (Saga et al., 1996) using antisense riboprobes to Hex1/Rps (Thomas and Beddington, 1996; Hermes et al., 1996), Otx2 (Simeone et al., 1993), Brachyury (T) (Herrmann, 1992), Six3 (Olive et al., 1995), Hnf3β (Ang and Rossant, 1994), Dkk1 (Glinka et al., 1998), Wnt3 (Liu et al., 1999), Wnt3a (Takada et al., 1994), Wnt8 (Bouillet et al., 1996), Frzb (Leys et al., 1997; Hoang et al., 1998), Sfrp1 (Hoang et al., 1998), Sfrp2 (Leimeister et al., 1998) and Cer1 (Belo et al., 1997).

**Scanning electron microscopy**

Dissected embryos were placed into Teflon baskets (Ted Pella, Redding, CA) and prepared for scanning electron microscopy by standard procedures. electron micrographs were taken at 10 kV on a Hitachi S4500 scanning electron microscope (Robards and Wilson, 1993).

**Detection of blood islands**

Embryos were fixed in 4% paraformaldehyde and washed in PBS. Staining of the blood islands was performed in 0.1% benzidine solution dissolved in PBS and 0.1% glacial acetic acid.

**Detection of primordial germ cells**

Embryos were fixed in 4% paraformaldehyde and washed in PBS, 70% ethanol and H2O. Primordial germ cells were stained in 5% veronal buffer, 0.6% MgCl2, 0.1% α-naphthylphosphate and 0.5% Nuclear Fast Red.

**RESULTS**

**Generation of Ldb1 null mutant mice**

Mice carrying a null allele of Ldb1 were generated through homologous recombination in ES cells. The genomic organization of the Ldb1 gene (Yamashita et al., 1998) and the vector used for gene targeting are shown in Fig. 1A. The targeting vector contained the neomycin-resistant gene, which replaced the coding region from exon 3 to exon 9 following homologous recombination. Two independent positive clones (Fig. 1B) were injected into C57BL/6 blastocysts to generate chimeric animals. Chimeric mice were crossed with CD1 females to obtain heterozygous offspring. Mice heterozygous for the Ldb1 mutation appeared normal and fertile. Heterozygous mice were intercrossed to obtain homozygous mutants. No viable mutants were found at birth (n=143), or at E16.5 (n=37). Homozygous mutants died at E9.5-E10.

**Ldb1 null mutant phenotype: defects in embryonic development**

Functional ablation of the Ldb1 gene led to a multitude of developmental defects along the anterior-posterior axis of the early mouse embryo. In comparison with wild-type (Fig. 2A), gastrulating mutant embryos showed a characteristic constriction at the embryonic-extraembryonic junction (Fig. 2B). The embryonic portion is generally smaller than that in the wild type. Mutant phenotypes were clearly visible at E8.5 and included anterior truncation, absence of heart formation
Role of the mouse Ldb1 gene in axial patterning

and loss of foregut indentation (Fig. 2E, compared with the controls shown in Fig. 2C,D). En2 expression that marks the midbrain-hindbrain boundary of wild-type embryos (Fig. 2F) is absent in the mutant (Fig. 2G,H) suggesting a disruption in midbrain and anterior hindbrain development. Also, the region of expression of Krox20, a hindbrain marker that marks rhombomeres r3 and r5 in the control (Fig. 2I), is abnormal in Ldb1 mutant embryos (Fig. 2J). The pattern of head truncation in Ldb1 mutant embryos was similar to that seen in the most severely affected Otx2 mutants (Acampora et al., 1995; Ang et al., 1996). However, in contrast to Otx2 mutants, the anterior region terminated in a convoluted neural plate structure implying a defect in the regional expansion of the neuroepithelium (Fig. 2L). Ldb1 mutants lack all rostral structures anterior of the otic vesicle (Fig. 2L). Columns of somites were often fused medially. Defective longitudinal extension of the neuroepithelium is the likely cause of the kinky and compressed shape of the neural tubes in the mutant embryos. Histological sections of E8.5 mutants at the trunk level show two neural grooves that are connected by a single neuroepithelial layer (Fig. 2L). Ldb1 mutants lack all rostral structures anterior of the otic vesicle (Fig. 2L). Columns of somites were often fused medially. Defective longitudinal extension of the neuroepithelium is the likely cause of the kinky and compressed shape of the neural tubes in the mutant embryos. Histological sections of E8.5 mutants at the trunk level show two neural grooves that are connected by a single neuroepithelial layer (Fig. 2L). A similar phenotype has previously been observed in Lim1 mutants (Shawlot and Behringer, 1995). Moreover, trunk duplication was observed visually in approximately 40% (24/56) of mutant embryos. Light and scanning electron microscopy revealed the presence of multiple rows of somites (Fig. 2N,O). The partial duplication of the Krox2 signal (Fig. 2I) suggests that posterior axis duplication extends to the rostral limit of mutant embryos. Although Ldb1 mutant embryos survived through stage E9.5, further development appeared to be arrested at E8.5. This may be a consequence of an extensive apoptotic cell death that occurs in the mesenchymal tissue of E8.5 mutant embryos (Fig. 2P,Q), thereby disrupting further tissue differentiation.

Ldb1 null mutant phenotype: defects in extraembryonic tissues

Defects in the development of extraembryonic tissues became apparent in Ldb1 null mutant embryos as they approached the neural-fold stage (E8.5). The yolk sac failed to expand around the fetus and a large portion of the anterior part of the embryo developed outside the yolk sac (Fig. 2O). The development of blood islands and primordial germ cells (PGCs) were also defective in the mutant yolk sac. Benzidine, specific for hemoglobinized erythroid cells, aided in the identification of vitelline blood vessels. In contrast to wild-type (Fig. 3A), mutant embryos lacked any sign of hematopoietic development (Fig. 3B). Identification of PGCs was based on their high alkaline phosphatase activity; these were well developed in the wild-type embryo at E7.5 (Fig. 3C) (see also magnified cells in the inset). However, cells showing this staining were either absent (Fig. 3D) or greatly reduced in Ldb1–/– mutants. Another mesoderm-derived structure, the allantois, was also defective and in a few instances entirely absent. Where present, the allantois had become largely detached from the wall of the exocoelomic cavity and was posteriorly displaced by E7.75 (compare Fig. 3E and F). In addition, it appeared to have lost contact with the amnion (Fig. 3F). By E8.5 the tissue had grown towards the chorion but failed to make contact with it.
Molecular analyses of the heart phenotype

The heart phenotype of embryos lacking Ldb1 gene function was characterized with the help of marker analyses performed at critical stages of heart development. Prospective cardiac mesodermal cells invaginate through the primitive streak and migrate and spread out together with cranial mesoderm. Subsequently the bilaterally symmetric cardiac precursors migrate and converge at the midline of the embryo to form the cardiac crescent. Nkx2.5, a homeobox gene, is expressed in the cardiogenic precursor cells that form the cardiac crescent at the headfold stage (Lints et al., 1993). A marked reduction in Nkx2.5 expression pattern was observed in Ldb1 knockout embryos suggesting that the mutant embryos fail to develop a proper cardiac crescent (compare Fig. 4A and B).

The Mesp1 mRNA is the earliest molecular marker for heart precursor cells known to date. At E7.0, expression of Mesp1 in Ldb1 null mutant embryos typically assumes an abnormal ‘inverted V’ shaped pattern, and the migration of the Mesp1-expressing mesodermal cells appears to be restricted to two lateral paths in proximal regions of the embryo (compare Fig. 4C and D). The proximal location of the Mesp1-expressing cells may suggest that the mutant embryos lack heart and craniofacial mesoderm that are derived from the distal part of the primitive streak and migrate along an anterior-distal path.
Role of the mouse Ldb1 gene in axial patterning

This defect in the generation and/or migration of the Mesp1-expressing heart progenitor cells may also account for the reduction in Nkx2.5 expression in the anterior cardiac field at E7.75 (not shown). Our marker analyses thus suggest that the heart phenotype in the Ldb1 null mutant is an early developmental defect affecting the generation and/or migration of the earliest recognizable heart precursor cells.

Molecular analyses of the anterior-posterior axis phenotype

We used an extensive array of markers to analyze defects in anterior-posterior axis formation in Ldb1 null mutant embryos. The posterior axis duplication observed in Ldb1 null mutant embryos was closely examined using Brachyury (T) as a molecular marker. In wild-type embryos, T expression was seen in the primitive streak throughout gastrulation (Fig. 5A), and in the streak and notochord at post-gastrulation stage E8.5 (Fig. 5D). In gastrulating mutant embryos, defects in streak formation were not always obvious (Fig. 5B). However, the twisted streak seen in some of these mutants suggested to us the presence of two overlapping primitive streaks (Fig. 5C). An abnormal V-shaped expression pattern of the Mesp1 gene in early ingressing mesodermal cells is observed in a stage-matched Ldb1 null mutant embryo (D; posterior is facing up). The arrows in B and D indicate the embryonic-extraembryonic junction.
failed to extend to the anterior-most aspect of the mutant embryos (Fig. 5K,L). It is likely that the duplicated expression patterns may have developed from duplicated nodes. This is supported by our Lim1/Lhx1 expression analysis that shows two distinct nodes in the mutant (compare Fig. 5M and N). Note that the probe used in this study confirms the published Lim1/Lhx1 expression pattern in wild-type embryos (Tsang et al., 2000). However, a small segment of the probe overlaps with the sequence of the closely related Lim5/Lhx5 gene. Therefore we cannot exclude the possibility that the expression pattern detected with this probe may correspond to Lhx5 gene. Nonetheless, our in situ analysis clearly reveals the presence of two nodes in Ldb1 mutant embryos.

Taken together our results suggest that the anterior truncation of Ldb1 null mutant embryos is the consequence of a disruption of Otx2 gene function in the head organizer tissue while the posterior axis duplication phenotype is the outcome of an early node partitioning event.

**Molecular mechanism of Ldb1 gene function**

Wnt pathways play an essential role in anterior-posterior axis patterning (McMahon and Moon, 1989; Popperl et al., 1997; Zheng et al., 1997; Liu et al., 1999). Anterior truncation and posterior duplication of the Ldb1 null mutant axis are reminiscent of phenotypes resulting from overactivation of Wnt pathways (Popperl et al., 1997; Borello et al., 1999). We therefore examined the potential role of Ldb1 in regulating Wnt. Wnt overactivation can be achieved either through upregulation of Wnt functions or downregulation of Wnt inhibitors. We focused our attention on those Wnt genes and inhibitors that are expressed in the early embryo and may play crucial roles in development of structures along the anterior-posterior axis. Our analysis of Ldb1+/– embryos failed to detect an increase in steady-state levels of Wnt3, Wnt3a or Wnt8 transcripts (data not shown). The Wnt inhibitor genes that are expressed during gastrulation include Dkk1 (Glinka et al., 1998), Cerberus-like (Cer1) (Belo et al., 1997) and the three secreted Frizzled-related genes Sfrp1 (Hogan et al., 1998),
Role of the mouse Ldb1 gene in axial patterning

Sfrp2 (Leimeister et al., 1998), and Frzb/Sfrp3 (Hoang et al., 1998). At E7.5, Frzb/Sfrp3 expression is seen in the primitive streak and prospective cardiac mesoderm of wild-type embryos (Fig. 6A) (Hoang et al., 1998). At this stage Sfrp1 expression is found in the ectodermal tissue located anterior to the migrating head process (Fig. 6C) (Hoang et al., 1998). By contrast, the expression domain of Sfrp2 is much broader, encompassing medial and distal embryonic tissues (Fig. 6E) (Leimeister et al., 1998). At late streak stages, Cer1 expression is seen in the AVE, axial mesoderm and in definitive endodermal cells of wild-type embryos (Fig. 6G) (Belo et al., 1997). Our analysis showed that expression of all five Wnt inhibitor genes tested is restricted in the mutants (Fig. 6B,D,F,H,I). While steady-state transcript levels of all Wnt inhibitors tested were reduced when compared to those of controls, the degree of reduction varied between individual mutants, as exemplified in Fig. 6I. Since Wnt proteins are known to act as posteriorizing agents (reviewed by Niehrs, 1999), the apparent downregulation of Wnt inhibitors suggests the possibility that the headless phenotype of the Ldb1 null mutants may result from overactivation of Wnt pathways along the anterior-posterior axis.

Detection of maternally deposited Ldb1 mRNA in the wild-type oocyte

The Drosophila Ldb1 homolog Chip is not only expressed zygotically but is also provided maternally (Morcillo et al., 1997). Maternal Chip mRNA is required for embryonic development before the onset of zygotic expression. Embryos lacking maternal mRNA are unable to form segments and die early in embryonic development while embryos lacking zygotic Chip are able to form segments. These embryos hatch but die as larvae (Morcillo et al., 1996). We have considered the analogous possibility that maternal Ldb1 mRNA may play a role in early mouse development. Our RT-PCR analyses of RNA samples extracted from eggs or zygotes before the onset of zygotic expression clearly showed deposition of maternal Ldb1 mRNA in early mouse embryos (Fig. 7). Although we were unable to detect the presence of maternal Ldb1 mRNA in E7.5 mutant embryos, the possibility remains that protein translated from maternally deposited mRNA may support early stages of development in the zygotic null mutants. Alternatively, or in addition, partial functional redundancy may exist between Ldb1 and Ldb2, the second member of the Ldb family (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997). It is possible that variables such as these may underlie the incomplete penetrance of phenotypes such as posterior axis duplication.

DISCUSSION

Ldb1 gene function is required for anterior patterning of the gastrulating embryo

The phenotype of the Ldb1 null mutant mouse embryo has uncovered major functions of the Ldb1 gene in the establishment of the primary anterior-posterior body axis. All mutant embryos show a disruption of anterior development as manifested by a truncation of anterior head structures and a lack of heart and foregut anlagen. The AVE, an extraembryonic tissue, has been strongly implicated in conferring initial rostral identity to the embryo before gastrulation (Thomas and Beddington, 1996). As gastrulation proceeds, head induction depends on crosstalk between cells of the AVE, the AME and the ANE that converge near the rostral end of the anterior-posterior axis (Shawlot et al., 1999; Tam and Steiner, 1999). The homeobox-containing transcriptional regulators Lmx1 and Otx2 play indispensable roles in head development as judged from the respective null mutant ‘headless’ phenotypes (Shawlot and Behringer, 1995; Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Ldb1 has been implicated to act as a cofactor for both of these transcription factors (Agulnick et al., 1996; Bach et al., 1997). Consistent with this we have demonstrated that in the head organizer tissue of Ldb1 null mutant embryos the Otx2 downstream pathway is affected. We have detected loss or reduction in mRNA expression levels of the Otx2 downstream genes Dkk1 and Hex1. Since Otx2 function is known to be required for induction of its own expression in the prospective anterior neuroectodermal tissue (Ang et al., 1994; Acampora...
et al., 1998), the observed reduction of the Otx2 expression domain in Ldb1 mutant embryos may further suggest a defect in Otx2 downstream pathways. Otx gene products can interact both with Ldb1 and with Lim1/Lhx1 (Bach et al., 1997; Nakano et al., 2000). Therefore, the head phenotype in Ldb1 mutant embryos may, at least in part, be attributable to a disruption of pathways controlled by Otx2 and/or Lim1/Lhx1.

**Ablation of Ldb1 causes posterior axis duplication**

The axis duplication that we observed in a majority of Ldb1 null mutant embryos is reminiscent of a similar phenotype observed in embryos that carry homozygous mutations at the fused locus (Gluecksohn-Schoenheimer, 1949; Perry et al., 1995). Axis duplication was also observed in Lim1/Lhx1 null mutant embryos. Here, as in our case, the duplication affected only the posterior region, resulting in a Y-shaped structure fused at the truncated anterior end (Shawlot and Behringer, 1995). Posterior axis duplication can be induced by grafting node tissue to a posterior-lateral position in the gastrulating mouse embryo (Beddington, 1994). Using the node marker Hnf3β and Lim1/Lhx1 (Ang and Rossant, 1994), we were able to determine that abnormal generation of a duplicated node structures is the underlying cause for posterior axis duplication in the Ldb1 mutant. The fact that functional ablation of either Ldb1 or Lim1/Lhx1 causes anterior truncation and can also induce partial posterior axis duplication may be taken as a genetic indication that an interaction of these two factors is essential for proper anterior-posterior axis development, from head to tail.

Furthermore, we noted that expression of several Wnt inhibitors is downregulated in Ldb1 mutant embryos. It is important to note that the function of Cer1 (Belo et al., 2000), and possibly that of other Wnt inhibitors as well, is dispensable during mouse embryonic development, suggesting that functional redundancy may exist. The severe phenotypic abnormalities observed in Ldb1 null mutant embryos may reflect the simultaneous functional obstruction of several Wnt inhibitors.

A number of recent studies have revealed a role of Wnt pathways in anterior-posterior axis formation (reviewed by Yamaguchi, 2001). Frequent posterior axis duplication is seen in transgenic mice over-expressing chicken Wnt8c (Pöpperl et al., 1997). Wnt3 has been implicated in anterior-posterior axis formation because null mutant embryos lack the primitive streak and do not form mesoderm and definitive endoderm (Liu et al., 1999). Loss-of-function analysis of Dkk1 demonstrated that this Wnt inhibitor is required for rostral development (Mukhopadhyay et al., 2001). Over-expression of the Wnt inhibitor Frzb during early mouse development leads to reduction in the size of caudal structures (Borello, 1999). Furthermore, Frzb interferes with the ability of Xwnt8 to induce double axes in Xenopus (Leyns et al., 1997; Wang et al., 1997). Whether proper regulation of Wnt inhibitors by Ldb1 (in conjunction with interacting LIM-homeodomain factors) is a prerequisite for regulation of Wnt pathways during anterior-posterior axis development remains to be determined.

**Ldb1 plays a critical role in heart formation**

In Ldb1 null mutant embryos heart development is compromised at early stages of development. Our data suggest that the Ldb1 gene function is essential for proper allocation of the Mesp1-expressing cardiac mesoderm to the heart field. In the chick, heart mesoderm is induced by the signals from the anterior endoderm. The heart precursor cells are in contact with presumptive anterior endoderm throughout their migration from the streak into the lateral plate (Garcia-Martinez and Schoenwolf, 1993). Abnormal migration of the heart mesoderm to a lateral-proximal region of the mutant embryo may therefore abolish inductive interaction of this tissue with anterior endoderm.

BMP signaling is known to be essential, but not sufficient, for heart formation (Schultheiss et al., 1997; Andree et al., 1998). Presently additional factors are being examined in the chick embryo. One of these is Crescent, a Frizzled-related protein that inhibits Wnt8c and is expressed in the anterior endoderm during gastrulation. Ectopic expression of Crescent in the posterior lateral plate mesoderm leads to development of ectopic heart tissue with beating cardiomyocytes. The Wnt inhibitor Dkk1 similarly induces heart-specific gene expression from anterior mesoderm when ectopically expressed. Furthermore, ectopic Wnt signals can repress heart formation from anterior mesoderm in vitro and in vivo. These results led to the conclusion that inhibition of Wnt signaling is required for heart development in the chick (Marvin et al., 2001). Similarly, Wnt antagonism initiates cardiogenesis in Xenopus (Schneider and Mercola, 2001). While this may also hold true for the mouse, there are presently no data that would support this notion. A vertebrate homolog of Crescent has not yet been identified. Furthermore, disruption of mouse Dkk1 gene function does not lead to a disruption of heart development (Mukhopadhyay et al., 2001). It is, of course, entirely possible that mammalian heart development requires the activity of more than one Wnt inhibitor and that the loss of Dkk1 gene function may be compensated by the activity of other Wnt inhibitors that remain to be identified. Finally, it is interesting to note that members of the GATA family of zinc finger transcription factors are required for mouse heart formation (Molkentin et al., 1997). GATA proteins have been detected in nuclear complexes that also contain Ldb1 (Wadman et al., 1997), in keeping with the possibility that a physical interaction of Ldb1 with GATA peptides may take place during heart development.

**Lack of Ldb1 gene function leads to defects in the development of extraembryonic structures derived from mesoderm**

Malformations of the yolk sac, amnion, primordial germ cells (PGCs) and allantois of the Ldb1 null mutant conceptus point to severe defects in posterior mesoderm formation. In general these phenotypes may reflect a defect in mesodermal cell migration even though primitive streak extension appears normal in the mutant.

The yolk sac plays a pivotal role in the delivery of nutrients to the embryo prior to fusion of the allantois to the chorion at E9.5 (Cross, 1994), and disruption of yolk sac function causes embryonic lethality (Bielska, 1999). In our mutants, the yolk sack failed to surround the entire embryo. This is reminiscent of malformations seen in the naturally occurring kinky mutant (Gluecksohn-Schoenheimer, 1949), and in Hnf3β (Foxa2) (Ang et al., 1996) and Gata4 (Molkentin, 1997) null mutants. However, the yolk sac phenotype of the Ldb1 null mutant is distinct from these because the posterior part of the embryo
develops within the yolk sac whereas the anterior region does not.

Within the Ldb1 null mutant yolk sac, the development of blood islands is compromised. In the wild-type controls, blood islands appear at E7.5 in the extraembryonic mesoderm of the yolk sac. They represent areas of primitive hematopoiesis and vasculogenesis that develop into a vascular network by E8.5. Defects of early hematopoiesis and the development of blood islands have also been observed in Bmp4 null mutants that, like our Ldb1 null mutants, are characterized by defective mesodermal differentiation (Winnier, 1995). Ldb1 can form a transcriptional complex with Lmo2, Tal1, Gata1 and E47. Functional ablation of the first three of these four proteins by targeted gene disruption results in severe defects in hematopoiesis (Wadman et al., 1997). The absence of blood islands in the Ldb1+/− mutant may thus be explained by assigning Ldb1 an essential role as a co-factor in early transcriptional events that initiate yolk sac hematopoiesis.

Also compromised in the Ldb1 null mutant yolk sac is the formation of the primordial germ cells (PGCs). These cells are characterized by a high alkaline phosphatase activity and can be visualized starting around E7.25 in the mesodermal part of the yolk sac at the base of the allantois, posterior to the primitive streak (Chiquoine, 1954; Lawson and Hage, 1994). Shortly thereafter, they follow a migration path along the hindgut endoderm and hindgut mesentery to the urogenital ridge where the gonad forms. Cell transplantation studies have suggested that PGC development requires a distinct micro-environment in the proximal epiblast (Tam and Zhou, 1996). Bmp4 (Lawson, 1999), Bmp8b (Ying, 2000), Hnf3β and Lim1/Lhx1 (Tsang, 2001) appear to be essential functional components of this environment, as their loss results in defects of PGC formation. Since Ldb1 is a known co-factor of Lim1/Lhx1 activity and PGC development is similarly compromised in Ldb1 and in Lim1/Lhx1 null mutants (Tsang, 2001), impairment of Lim1/Lhx1 activity is a likely cause of the PGC phenotype. In addition, Ldb1 activity may be required for the proper temporal and spatial expression of some or all of the other aforementioned factors known to play a role in PGC development. This is suggested by the fact that Bmp4 and Hnf3β were mis-expressed in the Ldb1 null mutant (data not shown).

A further defect in the development of extraembryonic tissues was seen in the allantois of the Ldb1 null mutant conceptus. The wild-type allantois is derived from posterior streak mesoderm and extends into the exocoelic cavity. Loss-of-function studies have implicated a number of genes in this process, including Brachyury (T) (Chesley, 1935), Hnf3β (Ang et al., 1994), VCAM-1 (Gurtner et al., 1995), Otx2 (Ang et al., 1996), Bmp4 (Lawson, 1999) and Lim1/Lhx1 (Tsang, 2001). The Ldb1 null mutants form an extra-coelomic cavity, indicating that posterior mesoderm has reached the correct extraembryonic location and has lined the exocoelom. However, after this initial step, growth of the allantois is arrested and fusion with the chorion does not occur.

We observed a marked constriction between the extraembryonic and extraembryonic portions of the E6.5–E7.5 Ldb1 null mutant. Similar constrictions have been observed in mutants that are homozygous for deletions in Hnf3β (Ang and Rossant, 1994; Weinstein et al., 1994), Lhx1/lim1 (Shawlot and Behringer, 1995; Shawlot et al., 1999), Otx2 (Ang et al., 1996) and Nodal (Varlet et al., 1997). During subsequent development, deficiencies in rostral structures are noted in all of these mutants. The constriction has been viewed as an indication of defective cell movement during gastrulation (Foley and Stern, 2001). Alternatively, it may reflect a failure of visceral endoderm to proliferate or to confer proper morphogenetic signals to the underlying embryonic ectoderm (Dufort et al., 1998). In the Ldb1 null mutant, the amnion fails to extend normally. As the volume of the epiblast and that of the exocoelom cavity increase rapidly after E6.5, a foreshortening of the amnion between these two regions of the conceptus may prevent lateral outgrowth in the mid-region, resulting in the observed constriction. Nonetheless, the embryonic and extraembryonic portions of the Ldb1+/− mutants are separated by the amnion, indicating that the initial formation of the posterior amniotic fold and the subsequent closure of the pro-amniotic canal occur properly. Around E7.25, coinciding with the malformation of the allantois and the PGCs, the expansion of the amnion becomes defective. The amnion consists of two layers, the amniotic mesoderm and the amniotic ectoderm. Our findings suggest that the failure of the amnion to extend appropriately is due to a defect in the ectodermal layer of the amnion (Fig. 3F), because the amniotic mesoderm forms pockets near the anterior and posterior end of the amniotic layer, while the ectoderm fails to expand properly. This indicates that there is an excess of tissue in the mesodermal layer relative to the ectodermal layer, making it unlikely that the mesodermal layer is primarily responsible for the constriction. However, as the allantois and PGCs are defective in the Ldb1 mutant, the development of the amniotic mesoderm, which is also derived from the posterior primitive streak, may nonetheless be defective as well.

**Conclusion**

Loss of zygotic Ldb1 function results in a multi-faceted phenotype that reveals a requirement of Ldb1 during early post-implantation development of the mouse embryo, including formation of the anterior-posterior axis, the anlage of the heart and the elaboration of major mesoderm-derived extraembryonic structures. The mutant phenotype very likely reflects the malfunction of a diverse array of transcription factors whose action depends on a functional Ldb1 co-factor. This, in turn, may affect major signaling events, including those mediated by the canonical Wnt pathway.

We thank Drs S. L. Ang, M. Kuehn, M. M. Shen and P. P. Tam for helpful suggestions; and Drs S. L. Ang, C. Chin, E. M. DeRobertis, M. Kuehn, G. Oliver, F. P. Lytten, Y. Saga and Y. Ying for materials. This work was partially supported by a Feodor Lynen Research
Fellowship from the Alexander von Humboldt Foundation, Germany, to A. T. and by NIH Grant HD36847 to K. M. Downs.

REFERENCES


