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## ***Isl1* and *Ldb* co-regulators of transcription are essential early determinants of mouse limb development**

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### **Abstract**

The developing limb has served as an excellent model for studying pattern formation and signal transduction in mammals. Many of the crucial genes that regulate growth and patterning of the limb following limb bud formation are now well known. However, details regarding the control of limb initiation and early stages of outgrowth remain to be defined. This report is focused on genetic events that pave the way for the establishment of a hindlimb bud. *Fgf10* and *Tbx* are crucial for early phases of limb bud initiation. Here we show that in the absence of *Isl1* or of *Ldb1/2* there is no hindlimb bud development. *Fgf10* expression in the bud mesenchyme is dependent on *Isl1* and its *Ldb* co-regulators. Thus, *Isl1* and the *Ldb* co-regulators of transcription are essential early determinants of mouse limb development. *Isl1/Ldb* complexes regulate *Fgf10* to orchestrate the earliest stages of hindlimb formation.

### **Introduction**

Following gastrulation, paraxial, intermediate and lateral plate segments arise and migrate out of the mesoderm of the primitive streak. Limbs develop from cells of the lateral plate mesoderm (Capdevila and Izpisua Belmonte, 2001; Zeller et al., 2009). These cells form bulges underneath the ectoderm and establish the limb-forming regions that are set within a precisely coordinated expression pattern of members of the family of Hox genes (Burke et al., 1995). Tbx transcription factors are essential for the specification of limb identity. *Tbx4* is associated with hindlimb development (Gibson-Brown et al., 1996), while *Tbx5* acts in development of the forelimb (Chapman et al., 1996). Following Tbx expression, *Fgf10* is expressed in the lateral plate mesoderm of the prospective limb bud mesenchyme. In vitro analysis has identified *Fgf10* as a direct target of *Tbx5* (Agarwal et al., 2003). *Fgf10* is essential both for limb bud initiation and for maintaining outgrowth (Ohuchi et al., 1997; Min et al., 1998; Sekine et al., 1999). Loss of *Fgf10* function abrogates hindlimb and forelimb development (Min et al., 1998; Sekine et al., 1999). *Fgf10* induces the expression of Fgf8 in the ectoderm. The outgrowth and development of the vertebrate limb bud depends on reciprocal interactions between *Fgf10* in the mesenchyme and a group of Fgf signals, including *Fgf4*, *Fgf8* and *Fgf17*, in the overlying apical ectodermal ridge (AER) (Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997; Lewandoski et al., 2000; Moon and Capecchi, 2000; Sun et al., 2002).

Several members of the LIM-homeobox (Lhx) gene family of transcription factors are essential for limb development. Earlier studies had shown that the Lhx genes *Lhx2*, *Lhx9* and *Lmx1b* play key roles in limb patterning and outgrowth (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Ng et al., 1996). Early limb bud formation is accompanied by the expression of *Lhx2* and *Lhx9*. *Isl1*, an additional Lhx gene, is expressed specifically in the early hindlimb bud of the mouse embryo, with a peak of high level of expression at E9.5 that declines rapidly during the following 24 hrs of limb bud development (Sun et al., 2007;

Tzchori et al., 2009). Simultaneous ablation of the partially redundant *Lhx2* and *Lhx9* genes leads to severe impairment of limb development which is further exacerbated by the simultaneous knockdown of the essential *Ldb1* co-regulator of Lhx gene activity in the limb bud mesenchyme (Tzchori et al., 2009). In the present study, we analyzed the role of the Lhx transcriptional machinery during the initiation of limb development in the mesenchyme of the emerging limb bud. We show that the Ldb1-Is11 complex is essential for hindlimb development and acts upstream of *Fgf10* in the limb bud mesenchyme. This is in agreement with recent findings by Kawakami et al. (Kawakami et al., 2011) who showed that *Is11* regulates hindlimb initiation upstream of *Fgf10*.

## Results and Discussion

Our analysis has been facilitated by the fact that *Ldb1* and *Ldb2*, the obligatory co-regulators of Lhx gene activity, are expressed during limb bud development (Tzchori et al., 2009). All genetic and molecular analyses to date support the idea that Ldb1 and Ldb2 proteins are functionally redundant, with Ldb1 showing higher expression levels throughout the embryo. *Ldb2* null mice are fertile and phenotypically normal, whereas *Ldb1* null embryos die shortly after implantation (Mukhopadhyay et al., 2003). To completely ablate *Ldb* activity in the developing limb, we resorted to conditional inactivation of *Ldb1* in an *Ldb2* null background (table 1). Floxed *Ldb1* (Tzchori et al., 2009) was inactivated in the limb bud mesenchyme by crossing in a T-Cre transgene that is expressed in the mesoderm emanating from the primitive streak, the source of the limb bud mesenchyme (Perantoni et al., 2005; Verheyden et al., 2005). The resulting mutant embryos did not develop beyond E14.5. Mutants were collected at specific stages of early limb development. In the control embryos, hindlimb buds become discernible at E10. In the *Ldb1/2* double mutants we failed to observe hindlimb development at this stage. There was no hindlimb bud at E14.5 either, thereby excluding the possibility that development was just delayed. Forelimb bud formation was not affected but further forelimb development was severely compromised (Fig. 1a,b). Skeletal staining of E14.5 mutants confirmed that hindlimb structures did not develop (Fig. 1c,d).

Forelimb development is not stopped but merely impaired by the inactivation of the obligatory *Ldb* co-regulators of Lhx gene activity. In our earlier study on Lhx gene involvement of limb patterning we noticed that not all cells in the early forelimb bud mesenchyme express T-Cre. As a consequence, part of this mesenchymal tissue escapes *Ldb1* ablation (Tzchori et al., 2009). The hindlimb develops about 24 hrs later (Capdevila and Izpisua Belmonte, 2001). At this stage of embryo development, sufficient levels of Cre enzyme may have accumulated in the hindlimb bud mesenchyme to quench most or all *Ldb1* activity.

Using a similar approach, we generated mice lacking *Is11* gene activity in the hindlimb bud mesenchyme via T-Cre mediated *Is11* gene inactivation (table 1). Of all the resulting *Is11* mutant embryos (n=>20) less than 30% survived to E10.5, whereas those heterozygous for the *Is11* defect appeared normal. E9-E10.5 embryos were collected. *Is11* mutant embryos were significantly smaller than the controls and lacked hindlimb buds (Fig. 2). Their forelimb bud development was indistinguishable from that of the controls, in keeping with the fact that *Is11* is selectively expressed in the hindlimb bud.

In a scenario where Is11 and the Ldb coregulators are part of a complex regulating early events in hindlimb development, one would expect very similar phenotypes in the respective knockdown mutants provided *Is11* and the *Ldb* co-regulators work in strict conjunction in the T-Cre targeted cells. However, the *Is11* mutants are much smaller than the *Ldb1/2* mutants, and they die earlier. In both cases, knockdown was mediated by T-Cre mediated

excision of the *Isl1* and the *Ldb1* target genes, respectively. Phenotypic discrepancies between the *Isl1* and the *Ldb1/2* mutants may be best explained by postulating that the floxed *Isl1* gene is more accessible than floxed *Ldb1* for T-Cre-mediated knockdown. Such differences in Cre access to individual floxed genes have been reported earlier (Vooijs et al., 2001; Birling et al., 2009). In addition or alternatively, differences in the half lives of *Isl1* and *Ldb1* gene products may have affected the respective mutant phenotypes (Vooijs et al., 2001). Finally, we cannot rule out the possibility that *Isl1* has functions that are independent of the co-regulator *Ldb*.

*Fgf10* is recognized as a key regulator that is indispensable for limb bud formation (Ohuchi et al., 1997; Min et al., 1998; Sekine et al., 1999). Early hindlimb bud development is curtailed in *Ldb1/2*, *Isl1* and *Fgf10* mutants alike. We failed to observe *Fgf10* expression in the mesenchyme of the *Ldb1/2* and *Isl1* mutant hindlimb bud fields (Fig. 3a,d and Fig. 4a,b). This places the activity of the *Lhx* transcriptional apparatus in the nascent hindlimb bud upstream of *Fgf10*. In the forelimb region of the *Ldb* mutants, *Fgf10* expression was severely impaired, whereas expression persisted in the *Isl1* mutants (Fig. 3b,d). Our data are in full agreement with an extensive study of hindlimb initiation by Kawakami et al. (2011) who showed that *Isl1* is required for the activation of the *beta catenin* pathway upstream of *Fgf10*.

*Isl1* is not expressed in the forelimb bud. Thus, expression of *Fgf10* in the forelimb bud is regulated by an as yet unknown *Isl1*-independent mechanism.

*Tbx4* expression in the hindlimb buds, as examined at E10, was noted in both *Ldb* and *Isl1* mutants (Fig. 3c,f and Fig 4c,d). Kawakami et al. (Kawakami et al., 2011) report significant but not complete reduction of *Tbx4* expression at E9.5 in the lateral plate mesoderm of *Isl1* mutants at E 9.5 and conclude that *Isl1* may participate in the regulation of *Tbx4* in the hindlimb field. The persistence of *Tbx4* expression in *Ldb1* and *Isl1* mutants that fail to form a hindlimb bud suggests that *Tbx4* is not a determining factor of hindlimb bud initiation. Nonetheless, earlier knockout studies provided firm evidence that *Tbx4* participates in hindlimb bud development (Naiche and Papaioannou, 2003). The pathways that link *Ldb1*, *Isl1* and *Tbx4* during early phases of hindlimb bud development remain to be determined.

We conclude that *Isl1* is indispensable for early hindlimb bud formation and that its action in the bud mesenchyme is abrogated either by *Isl1* mutation or by removing the obligatory *Ldb* co-regulators of this *Lhx* gene. *Fgf10* expression in the hind bud mesenchyme is lost if the function of *Isl1* or its *Ldb* co-regulators are curtailed. Both are thus firmly associated with the earliest events in hindlimb development that precede *Fgf10* induction in the mesenchymal cells of the nascent limb bud.

## Experimental Procedures

### Mice strains and genotyping

The *Ldb1* conditional mutant mouse has been described previously (Zhao et al., 2007; Tzchori et al., 2009). Primer sequences for genotyping *Ldb1* wt alleles (350 bp) and *Ldb1* floxed alleles (400 bp) were as follows: *Ldb1S* 5'-CAG CAA ACG GAG GAA ACG GAA GAT GTC AG-3' and *Ldb1AS* 5'-CTT ATG TGA CCA CAGC CAT GCAT GCAT GTG-3'.

*Ldb2* knockout mice (unpublished) did not have any discernible phenotype. They were healthy, fertile, and lived a normal lifespan. Primer sequences for genotyping the *Ldb2* wt and null alleles were *Ldb2 F2* 5'- CAG ACT GCT CAT CAC ACA CTT GTT GAT-3',

Ldb2 R2-5' CTC AAG AAA TCC AAG GGG TGT TAC TTA-3', SCNEOR 5'-GTC CAG ATC ATC CTG ATC GAC A-3' and Neo3 5'-GAT CCC CTC AGA AGA ACT CGT-3', respectively. Primer sequences for genotyping the wt *Isl1* allele (452 bp) and the floxed *Isl1* allele (519 bp) were *Isl1* KC F 5'-GGT GCT TAG CGG TGA TTT CCT C-3' and *Isl1* KC R 5'-GCA CTT TGG GAT GGT AAT TGG AG-3', respectively. The T-Cre allele was genotyped using the primer sequences T-cre F 5'-CCA TGA GTG AAC GAA CCT GG-3' and T-Cre R 5'-GGG ACC CAT TTT TCT CTT CC-3'.

### Skeletal analysis

Skeletal staining was performed as described previously (Tzchori et al., 2009), with minor modifications. Briefly, E14.5 embryos were dissected in PBS and fixed in 95% ethanol for 1-3 days. Embryos were stained in alcian blue overnight, washed three times in 90% ethanol, incubated in 1% KOH for 2 h, and stained in alizarin red overnight. Embryos were washed in 2% KOH for 3 days or until they were clear, transferred to 20% glycerol in KOH overnight and stored in 50% glycerol in 50% ethanol.

### In Situ Hybridization

Whole mount RNA in situ hybridization was performed as described previously (Mukhopadhyay et al., 2003) with minor modifications. The time of proteinase K digestion was 15 minutes for E10.5 embryos and 12 minutes for E9.5 embryos. Exposure to BM purple was overnight at 4°C. When the reaction was complete, the embryos were washed three times in PBT and refixed in 4% PFA for 1 h. Embryos were transferred to 50% then 70% glycerol.

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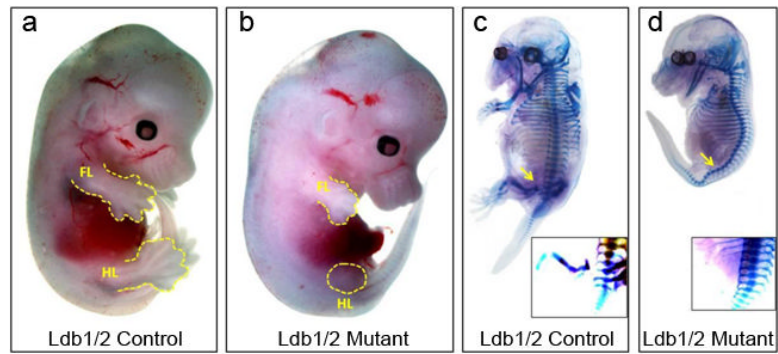
The *Isl1* conditional mutant mouse was kindly provided by Dr. Lin Gan, University of Rochester School of Medicine and Dentistry. The *Fgf10* and *Tbx4* probes were generous gifts of Dr. Yingzi Yang, NIH/NHGRI. We thank Dr. Karl Pfeifer and Dr. Sohyun Ahn for helpful discussions. This research was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

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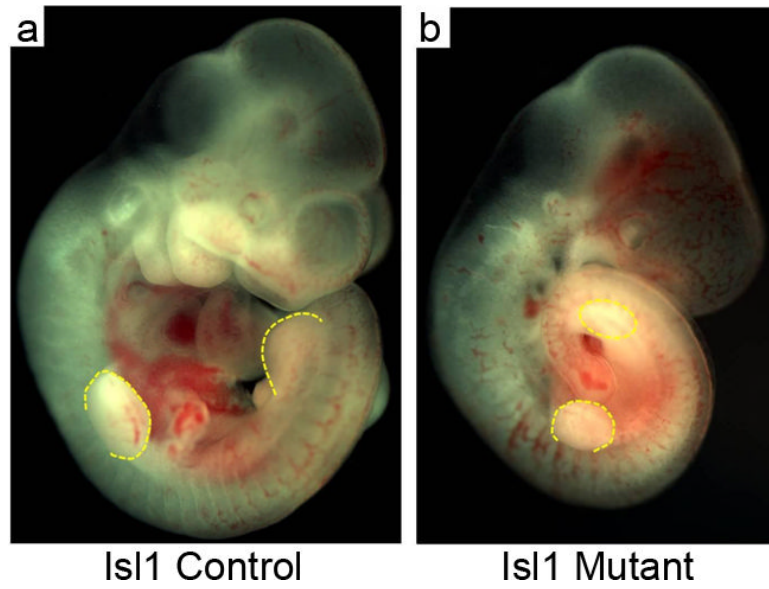
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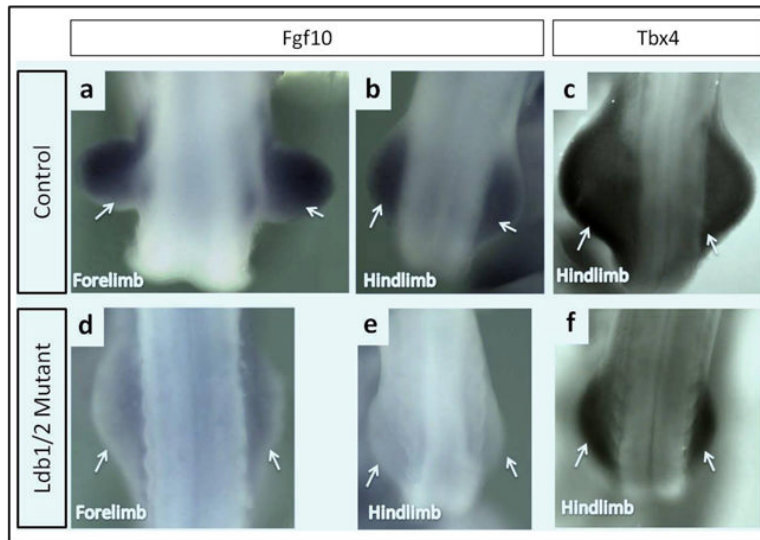


**Fig. 1.** *Ldb1/2* mutant embryos lack hindlimb formation. *Ldb1*<sup>fl/+</sup>;*Ldb2*<sup>+/-</sup> control embryo (a) compared to *Ldb1*<sup>fl/fl</sup>;*Ldb2*<sup>-/-</sup>;*Tcre* mutant embryo (b) at E13.5. Skeletal structure of a corresponding pair of control *Ldb1*<sup>fl/+</sup>;*Ldb2*<sup>+/-</sup> (c) and *Ldb1*<sup>fl/fl</sup>;*Ldb2*<sup>-/-</sup>;*Tcre* mutant embryos at E14(d). Embryos were stained with alizarin red and alcian blue. FL, forelimb; HL, hindlimb.

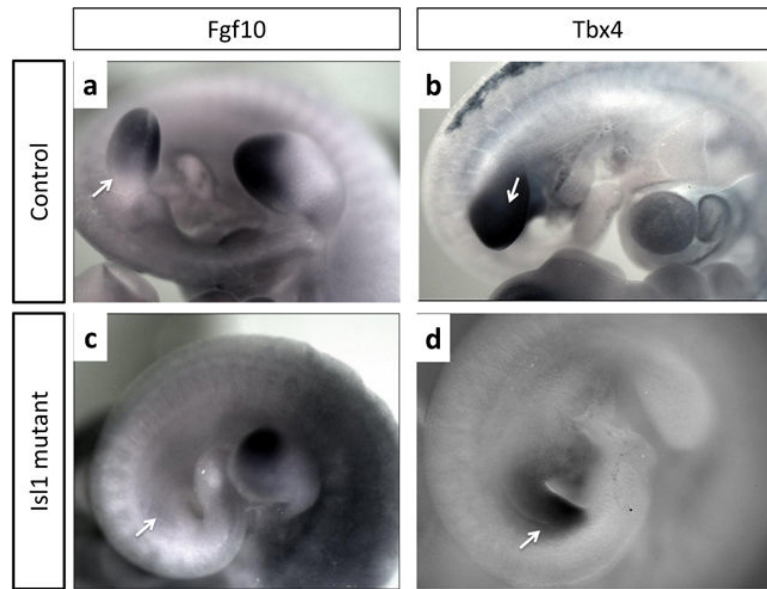


**Fig. 2.** *Isl1* mutant embryos lack hindlimb formation. *Isl1*<sup>fl/+</sup> control embryo (a) compared to *Isl1*<sup>fl/fl</sup>; *Tcre* mutant embryo (b) at E10.5.





**Fig. 3.** *Fgf10* expression is absent in the hindlimb bud of *Ldb1/2* mutant embryos. RNA whole mount In situ hybridization. *Fgf10* expression in the forelimb and hindlimb buds (a,b) of *Ldb1<sup>fl/+</sup>;Ldb2<sup>+/-</sup>* control compared to the expression in the forelimb and hindlimb buds (d,e) of *Ldb1<sup>fl/fl</sup>;Ldb2<sup>-/-</sup>;Tcre* mutant embryos at E10. *Tbx4* expression in the hindlimb buds of *Ldb1<sup>fl/+</sup>;Ldb2<sup>+/-</sup>* control (c) and *Ldb1<sup>fl/+</sup>;Ldb2<sup>+/-</sup>* mutant embryos (f) at E10



**Fig. 4.** *Fgf10* expression is absent in the hindlimb bud of *Is1* mutant embryos. RNA whole mount In situ hybridization. *Fgf10* expression in the hindlimb buds (a) of *Is1*<sup>fl/+</sup> control (a) compared to that of the *Is1*<sup>fl/fl</sup>;Tcre mutant (b) embryos at E10.5. *Tbx4* expression in the hindlimb buds of *Is1*<sup>fl/+</sup> control (c) and *Is1*<sup>fl/fl</sup>;Tcre mutant embryos (d) at E10. Hindlimb position is indicated by arrows

**Table 1**

<b>Name</b>	<b>Mouse Line</b>	<b>Cross</b>
<b>Ldb1/2 Control</b>	Ldb1 <sup>fl/fl</sup> ;Ldb2 <sup>-/-</sup>	Ldb1 <sup>fl/fl</sup> ; Ldb2 <sup>+/+</sup> X Ldb1 <sup>+/+</sup> ;Ldb2 <sup>-/-</sup>
	Ldb1 <sup>fl/+</sup> ;Ldb2 <sup>+/-</sup> ;Tcre	Ldb1 <sup>fl/fl</sup> ;Ldb2 <sup>-/-</sup> X Ldb1 <sup>+/+</sup> ;Ldb2 <sup>+/+</sup> ;Tcre
<b>Ldb1/2 double mutant</b>	Ldb1 <sup>fl/fl</sup> ;Ldb2 <sup>-/-</sup> ;Tcre	Ldb1 <sup>fl/fl</sup> ;Ldb2 <sup>-/-</sup> X Ldb1 <sup>fl/+</sup> ;Ldb2 <sup>+/-</sup> ;Tcre
<b>Isl1 Control</b>	Isl1 <sup>fl/+</sup> ;Tcre	Isl1 <sup>fl/fl</sup> X Isl1 <sup>+/+</sup> ;Tcre
<b>Isl1 mutant</b>	Isl1 <sup>fl/fl</sup> ;Tcre	Isl1 <sup>fl/fl</sup> X Isl1 <sup>fl/+</sup> ;Tcre

Ldb1<sup>fl/fl</sup>, Ldb2<sup>-/-</sup>, Ldb1<sup>fl/fl</sup>;Ldb2<sup>-/-</sup>, Tcre, Ldb1<sup>fl/+</sup>;Ldb2<sup>+/-</sup>;Tcre, Isl1<sup>fl/fl</sup>, and Isl1<sup>fl/+</sup>;Tcre mice appear viable and fertile.